INTERACTIONS BETWEEN SPONGES AND MARINE BACTERIA AS A ROUTE TO THE DISCOVERY OF NOVEL BIOACTIVE COMPOUNDS

By

Noraznawati Ismail

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School of Life Sciences

Heriot-Watt University

Edinburgh

EH14 4AS

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ABSTRACT

The bacterial community architecture associated with four species of sponges, *Halichondria panicea*, *Suberites domuncula*, *S. carnosus* and *Pachymatisma johnstonia* was investigated using culture-dependent and culture-independent strategies. Marine agar was found to be the best of several media used for cultivation of culturable bacteria associated with sponges. Molecular methods, including denaturing gradient gel electrophoresis (DGGE), 16S rDNA cloning and sequence analysis suggested a bacterial community different from that identified using culture-dependent methods. DGGE can provide a profile of the whole community of the sponge and facilitate screening of large-scale samples. 90% of the bacteria associated with these four sponges were sponge species-specific. *S. carnosus* was also transferred to an aquarium to study kinetic changes of sponge-associated bacterial communities. DGGE analysis showed the consistent presence of some particular bands suggesting the continued presence of species of symbiotic bacteria. Four *Bacillus* species (*B. licheniformis* SC-43, *B. subtilis* SD-8, *B. pumilus* HP-48 and *B. cereus* HP-22) isolated from the sponges exhibited antagonistic activity against isolates of Gram-positive bacteria obtained from the same sponges. All strains tested were active against *Micrococcus luteus*, strain HP-5/6 isolated from *H. panicea*. This suggests that HP-5/6 can be used in the laboratory as a sensitive indicator of activity. A comparison of several media found Nutrient Agar/ Broth containing glycerol and iron (NGF) to be the best medium tested for antimicrobial compound production. *B. licheniformis* (SC-43), *B. subtilis* (SD-8), and *Pantoea* sp., SC-AF, in the presence of glycerol and ferric ion, could produce antimicrobial compounds when grown within biofilms; however, the corresponding shaken flask cultures could not. This effect could be related to oxidative stress defence responses. *Pantoea* sp., SC-AF produced several antimicrobial compounds active against *M. luteus*, HP-5/6 which were different from previously reported Pantocin antimicrobials. In addition, *Pantoea* sp., SC-AF produced 'jelly-like' extracellular polysaccharide (EPS) on NGF and on the nylon membrane in Air-membrane surface bioreactor (AMS) cultures, along with the production of antimicrobial compounds. Only fructose and cellobiose after acid lysis of EPS of *Pantoea* sp., SC-AF have been identified. In addition, my study confirmed that sponges accommodate large amounts of uncultured bacteria, whose metabolic capability cannot be explored without cultivation. New cultivation strategies should be investigated and biofilm-based culture techniques incorporated in the future search for novel antibiotics.

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DEDICATION

Dr. Liming Yan

DECLARATION

I, Noraznawati Ismail, hereby declare that I am the author of this thesis. All work described here in this thesis is my own, except where stated in the text. The work presented here has not been accepted in any previous application for a higher degree. All the sources of information have been consulted by myself and are acknowledged by means of references.

Noraznawati Ismail

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ABBREVIATIONS

°	degrees
°C	degrees Celcius
µg	micrograms
µl	microlitres
µm	micromole
µM	microMolar
%	percent
AHL	Acyl-Homeserine lactone
ASW	artificial seawater
AI	auto-inducer
B.	*Bacillus* sp.
BLAST	basic logical alignment search tool
DGGE	denaturing gradient gel electrophoresis
dNTP (s)	deoxyribonucleoside 5'-triphosphate
DNA	deoxyribonucleotide
D_2O	deuterium water
dH_2O	distilled water
E. coli	*Escherichia coli*
EDTA	diaminoethanetetra-acetic acid
EMBL	European Molecular Biology Laboratory
EPS	Extracellular polysaccharide
h	hour
HP	*Halichondria panicea*
MHz	mega hertz
Mg	milligrams
min	minute
mw	molecular weight
kDa	kiloDalton
no	number

nt	nucleotide
nm	nanometer
PCR	polymerase chain reaction
PJ	*Pachimatisma johntonia*
r	reverse
16S rDNA	16 subunit ribosomal DNA
rpm	rotation per minute
sp.	species
subsp.	subspecies
SD	*Suberites domuncula*
SC	*Suberites carnosus*
Taq	*Thermus aquaticus*
TBE	Tris-Boric acid-EDTA
U	unit
UV	ultraviolet radiation
vol	volume
v/v	volume volume ratio
w/v	weight volume ratio

CHAPTER 1 INTRODUCTION

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1.1 CONCISE BIOLOGY OF SPONGES

Sponges (phylum *Porifera*) are among the oldest metazoan multicellular animals, with a fossil record dating back more than 580 million years to the Precambrian (Müller, 1995; Matsunaga *et al.*, 2000). More than 10, 000 species have been described so far (Hooper *et al.*, 1999; Zhang *et al.*, 2003). Sponges contribute significantly to, and sometimes even dominate, the tropical reef fauna in terms of biomass, but are also found in polar and deep oceans, freshwater, lakes and streams. Eighty-five percent of the 6000 formally described living species belong to the class *Demospongiae* (demosponges), with the other species being represented by the classes *Hexactinellida* (glass sponges) and *Calcarea* (calcareous sponges) (Hooper and van Soest, 2002).

1.1.1 Sponge structure

Sponges are currently divided into three different classes, *Demospongiae*, *Hexactinellida* and *Calcarea,* based on the nature of their skeleton (Vacelet and Donadey, 1977; Hooper and van Soest, 2002). With the formation of an inorganic skeleton the body plan of metazoans is defined and its orientation is fixed. In most sponges their solid support, the spicules, is composed of hydrated, amorphous and noncrystalline silica (SiO_2/H_2O) or calcium carbonate ($CaCO_3$) depending of the class of the sponges. Demospongiae form the largest class, comprising approximately 85 % of all species, in which the skeleton is composed of siliceous spicules that are often supplemented by organic collagenous fibres (Hooper and van Soest, 2002).

Calcareous sponges possess a skeleton that is composed entirely of calcite spicules (Jones, 1970), while Hexactinellida, which are primarily deep-water sponges, have a skeleton that is built of six-rayed (hexactinal) siliceous spicules (Reiswig, 1979). The secretion of spicules occurs in specialized cells, the sclerocytes, where silica and calcite is deposited in an organized way (Bergquist, 1978). The spicules and collagen fibres form a strong network in the mesohyl that comprises the space between the exopinacoderm and the

endopinacoderm. In addition to collagen fibres, the mesohyl comprises of galectins, fibronectin-like molecules, dermatoportin and polysaccharides (Schutze *et al.*, 2001). These macromolecules form the extracellular matrix, which provides the platform for specific cell adhesion as well as for signal transduction and cell growth. Because of these functions, the extracellular matrix plays vital roles in digestion, gamete production, transport of nutrients and waste products by archaeocytes that can move freely through the mesohyl (Müller *et al.*, 2004). Although many different cell types are present in sponges, only two types of organ-like structures can be defined: pinacocytes forming a pinacoderm and choanocytes forming choanocyte chambers (Fig 1.1). The other cell types are scattered through the mesohyl (Hooper and van Soest, 2002).

Archaeocytes are the most prominent cells in the mesohyl. Besides transport through the sponge and digestion of nutrients, they have the capacity to differentiate into any other cell type. They provide a regulatory mechanism establishing and maintaining the equilibrium between different cell types. Some capacity for further development is retained by choanocytes, which can form gametes and by collencytes, which can become pinacocytes or myocytes (Sipkema, 2004). However, the micro-architecture of the aquiferous system is diverse and complex (Reiswig, 1975) and depends on the life-history strategy of the sponge (Turon *et al.*, 1997). The structural organization of the aquiferous system affects the clearance rates of the sponge (Turon *et al.*, 1997) and therefore it can be assumed that numbers of bacteria and retention in the aquiferous system of sponges depends on the different organization structure of each sponge species. Lastly, there are also many cell types containing small granules or vesicles. All these cells types can be grouped as granulocytes.

A large number of different functions are attributed to the different granulocytes. They have been found to be a depot of certain secondary metabolites (Thompson *et al.*, 1983), unconventional sterols (Lawson *et al.*, 1988), pigments (Rutzler, 1990) or glycogen (Bergquist, 1978). In addition, some granulocytes release components that compose the mesohyl, like mucous (Brusca and Brusca, 1990) or lectins (Bretting *et al.*, 1983). Moreover, there are many more sponge-cell types but their roles are not specialized.

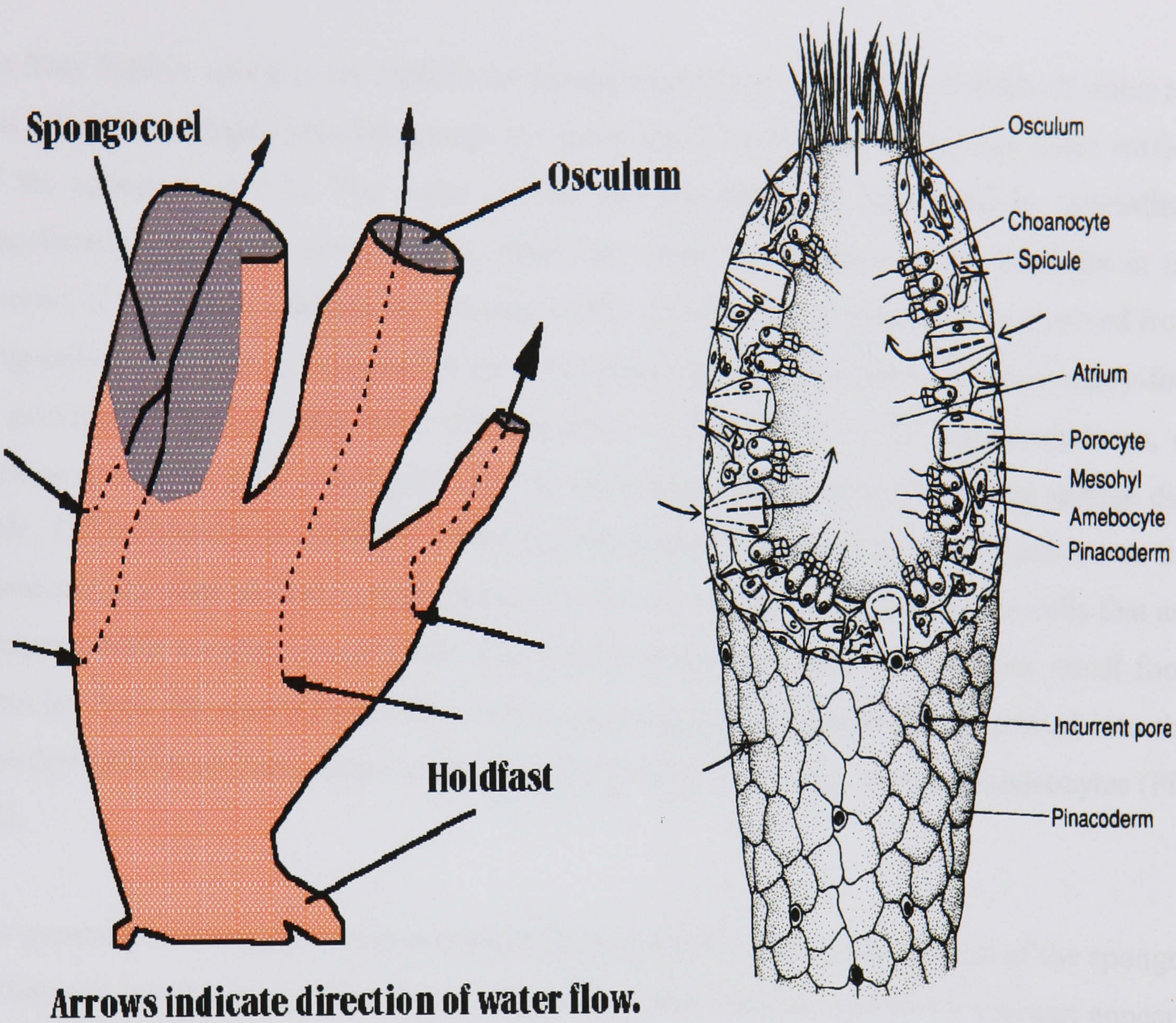


Figure 1.1 General morphology of a sponge (Porifera) (www.ucmp.berkeley.edu/porifera)

1.1.2 Physiology of sponges

As filter feeders sponges are capable of turning over many thousands of litres of water per day. Water is pumped into the sponge via many small canals that start at the outer surface of the sponge (Fig 1.2). The water current into the sponge is generated by specialized flagellated cells, called choanocytes, which are clustered in choanocyte chambers in the interior of the sponge (Brusca and Brusca, 1990). The feeding structures have evolved from originally respiratory structures, but their pumping capacity has had to increase many-fold to provide the sponge with sufficient quantities of food. Prokaryotic microorganisms, as well as nano- and pico- eukaryotes, are the most important components of the sponge diet (Pile, 1997; Ribes *et al.*, 1999). A more ingenious trap is required to retain nutrients in the processed seawater as their concentration is very low. Choanocytes are sponge cells that are equipped with a collar of microvilli that surrounds the flagellum to withdraw small food particles from the passing seawater. Following capture, food particles are moved into the mesohyl interior and are phagocytosed by amoeboid sponge cells, termed archaeocytes (Fig 1.3).

It is generally assumed that archaeocytes distribute the nutrients over the rest of the sponge, as they can travel through the sponge (Simpson, 1984). Particle uptake by sponges appears to be highly efficient but probably largely unselective for a given particle size range (Michin, 1900; Simpson, 1984). Dissolved oxygen is taken up via inefficient diffusion inside the canals and choanocyte chambers (Jorgensen *et al.*, 1986). Furthermore, food particles can be taken up by exo- and endopinacocytes that cover the outside of the sponge and the canals and the surfaces inside the sponge (Reiswig, 1975). Moreover, it has been suggested that sponges are capable of absorbing dissolved organic nutrients directly from the water (Reiswig, 1971; Yahel *et al.*, 2003).

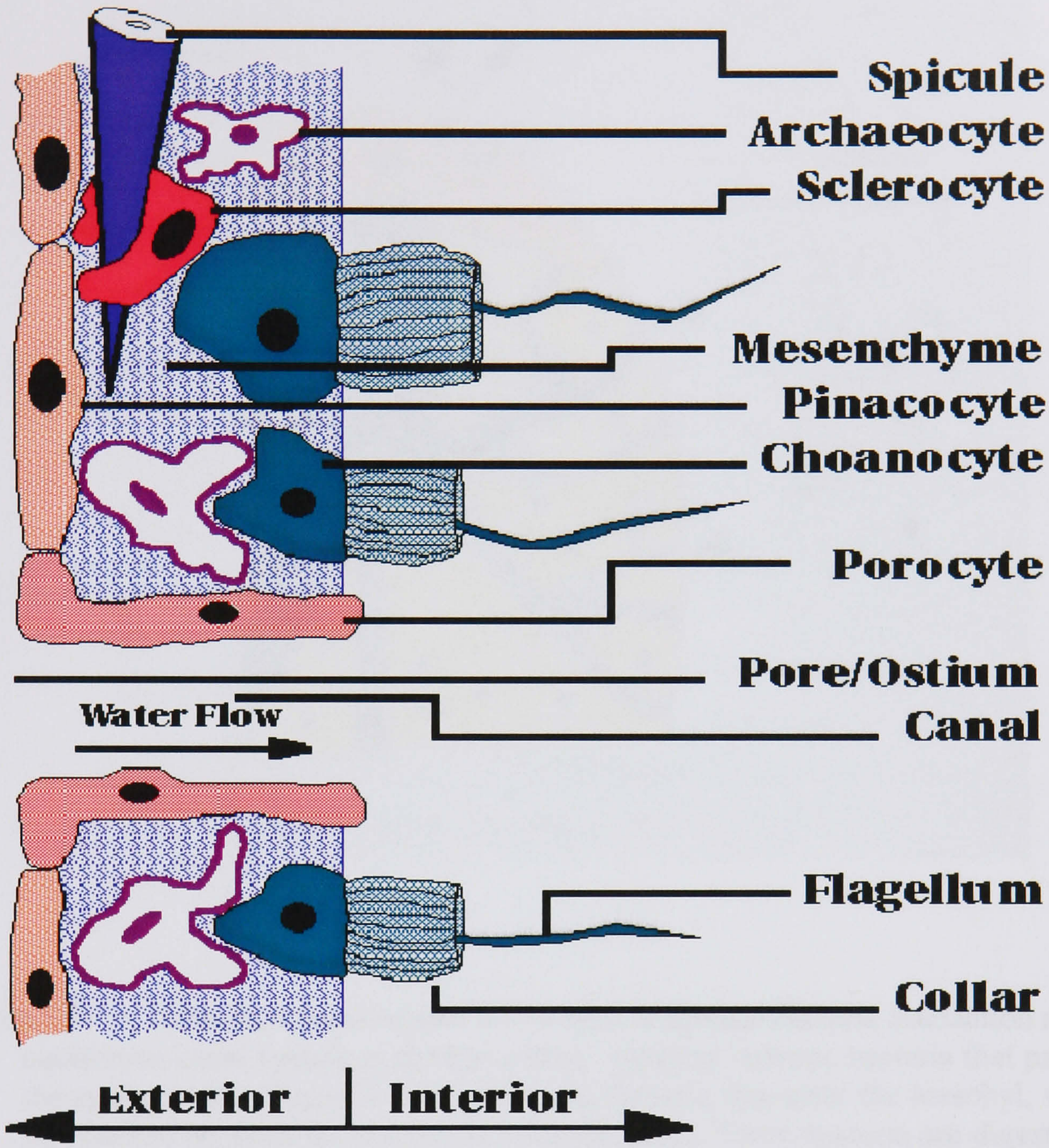


Figure 1.2 Microscopic view of a sponge (Porifera)(www.ucmp.berkeley.edu/porifera)

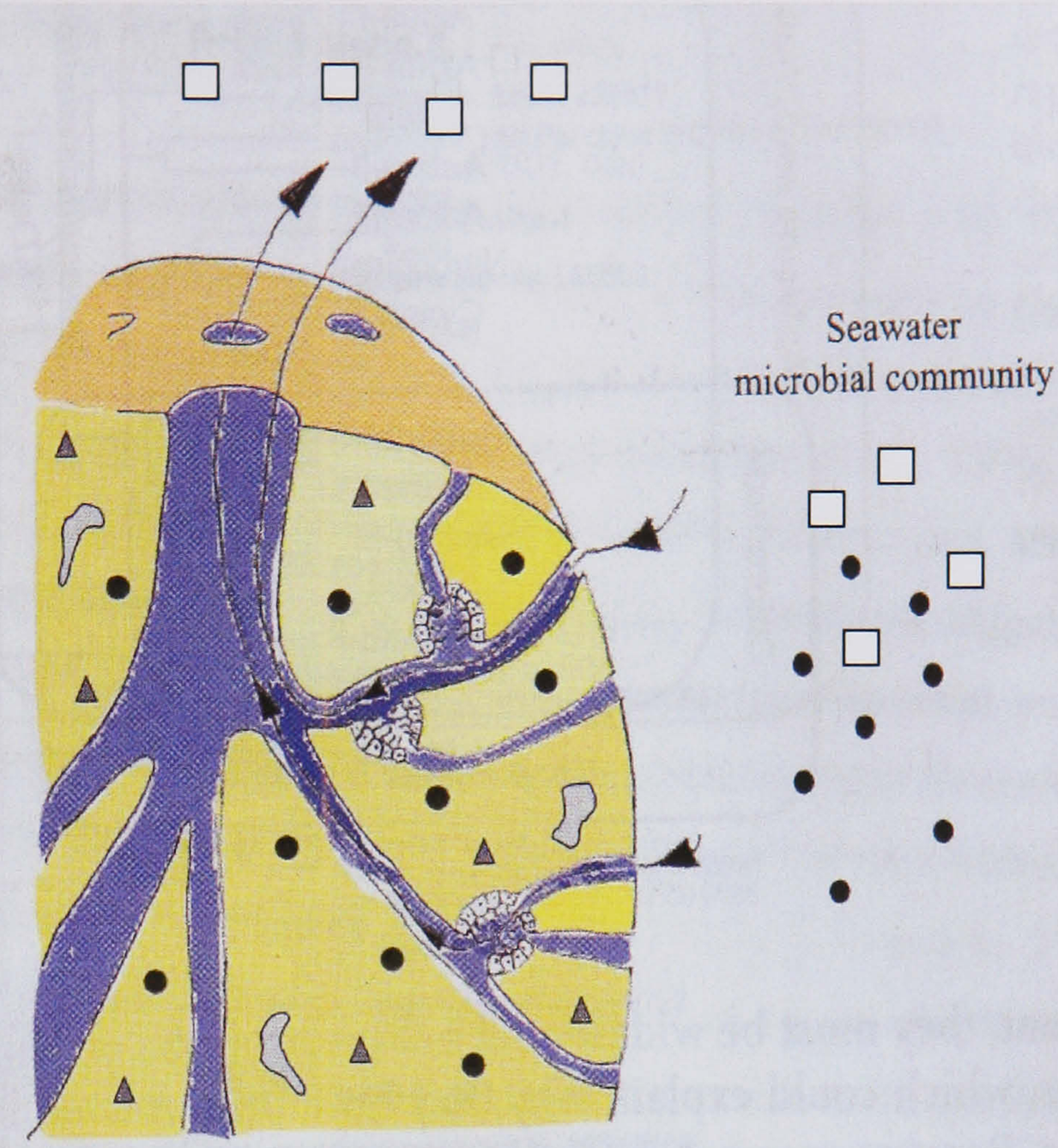


Figure 1.3 Schematic representation of a typical sponge-bacteria interaction model. The aquiferous canal system is shown in blue. Squares indicate bacteria that pass through the sponge unprocessed. Circles represent bacteria that enter the mesohyl, where their persistence depends on individual turnover times. Some bacteria are digested quickly serving as a food source, others persist for long times possibly due to unusual membrane structures. Triangles represent bacteria that have been transmitted via the reproduction stages and that are probably absent from seawater (Hentschel *et al.*, 2006).

1.2 SPONGE-BACTERIA ASSOCIATIONS

The unique nature of the sponge-microbe association, together with the ecological and biotechnological importance of the sponges, makes marine sponges an ideal system for the study of microbial diversity, evolution of the marine eukaryote-microbe association, and microbial dispersal associated with bioinvasions (Carballo *et al.*, 1996; Gili and Coma, 1998; Osinga *et al.*, 2001; Carballo and Naranjo, 2002; Thakur and Müller, 2004). The bacterial load in sponges seems proportionally correlated with the irrigation status of the sponge. Sponges with a poor irrigation system contain high bacterial numbers while the well-irrigation sponges have fewer bacteria within their mesohyl (Vacelet and Donadey, 1977; Wilkinson, 1978b). The presence of large numbers of bacteria within marine sponges was first established by microscopic studies (Vacelet and Donadey, 1977; Wilkinson, 1978c). While early work indicated high microbial diversity and the presence of unusual microorganisms, it was not until the application of molecular tools, specifically 16S rDNA gene library construction, fluorescent *in situ* hybridization (FISH), and denaturing gradient gel electrophoresis (DGGE), that more precise insights into microbial community composition could be gained.

Early studies determined the association of bacteria with sponges based on bacterial morphology and recognized three types of associations of bacteria with sponges (Vacelet and Donadey, 1977; Wilkinson, 1978a, 1978b, 1978c). The first group contained bacteria similar to those of ambient seawater and not specific to sponges, the second group contained intracellular bacteria that are specific to sponges, and the third group contained bacteria within the mesohyl, also specific to sponges. The characteristic features of the bacterial cell wall (Gram-positive or Gram-negative) and internal membrane structures also helped to identify several bacterial groups using transmission electron microscopy. Despite the fact that sponges feed on microorganisms, large numbers of extracellular bacteria populate the mesohyl matrix of many demosponges. These types of sponge have been termed 'bacteriosponges' or 'high-microbial-abundance sponges' (Vacelet and Donadey, 1977). However, the mesohyl of other sponges that coexist in the same habitat essentially support low abundances of microorganisms. In the bacteriosponges, bacterial population

densities may reach 10^8-10^{10} bacteria per gram of sponge wet weight, exceeding seawater concentrations by 2-4 orders of magnitude, whereas in the low-microbial-abundance sponges, they are within the range of natural seawater (10^5 – 10^6 bacteria per gram of sponge wet weight) (Hentschel *et al.*, 2006). Because the high-microbial-abundance sponges are typically larger than their low-microbial-abundance counterparts whose mesohyl is essentially devoid of bacteria, it is tempting to speculate that the presence of internal microbial biomass contributes to their large size. The Caribbean great barrel sponge, *Xestospongia muta,* serves as a suitable example to illustrate this point. As the largest known sponge species, *X. muta* may reach 2 m in height. With a bacterial population density of 8 x 10^9 microorganisms per gram of wet weight a single individual, conservatively estimated at 10 kg wet weight, will hold a bacterial population size of nearly 10^{14} microorganisms. These numbers underline the ecological relevance of sponge-associated microbiota for tropical reef ecosystems (Hentschel *et al.*, 2006).

The microbial distribution within a typical sponge residing in the photic zone follows a general pattern (Hentschel *et al.*, 2003). The outer, light-exposed layers are populated by photosynthetic microorganisms, while the internal mesohyl contains a complex mixture of heterotrophic and probably also autotrophic bacteria (Rutzler, 1985; Wilkinson, 1992). The vast majority of microorganisms are located extracellularly in the mesohyl matrix, where they appear to be homogenously mixed. In some sponges bacteria are found within host bacteriocytes and even within the host nuclei (Vacelet and Donadey, 1977). The association of bacteria in sponges has been demonstrated by several studies (Table 1.1). It is clear from this table that various microorganisms have evolved to reside in sponges, including a group of bacteria from α,δ, and γ-proteobacteria, *Pseudomonas* or *Alteromonas* sp., and *Bacillus* sp. Transient bacteria can also be trapped within the vascular system or attached to the sponge surface during filtration. Bacteria embedded in sponge mesohyl tissue were first described in electron microscope studies (Levi and Levi, 1965). Microorganisms detected so far in sponges include archaea, heterotrophic bacteria, cyanobacteria, red and green algae, dinoflagellates, and diatoms (Webster *et al.*, 2001; Hentschel *et al.*, 2002; Müller, 2003).

Table 1.1: Recent reports describing sponge-bacterial associations

Sponge species/class	Associated bacteria	References
Theonella swinhoei/ Demospongiae	*Candidatus, Entotheonella, palauensis*	(Faulkner *et al.*, 2000)
Chondrosia reinformis/ Demospongiae	α,δ,γ -*proteobacteria*	(de la Fuente *et al.*, 2003)
Dysidea fragilis/ Demospongiae	*Pseudomonas*/ *Alteromonas* sp.	(de Rosa *et al.*, 2001)
Rhopaloeides odorabile/ Demospongiae	Actinobacteria *Cytophaga/flavobacteria* Green non-sulphur bacteria Green sulphur bacteria	(Webster and Hill, 2001; Webb and Maas, 2002)
Aplysina aerophoba/ Demospongiae	*Bacteriodes* sp.	(Bohm *et al.*, 2001)
Mycale (carmia) *hentscheli*/ Demospongiae	*Cyanobacterium stanieri* *Prochloron* sp. *Synechocyctis* sp.	(Webb and Maas, 2002)
Halichondria japonica	*Bacillus cereus*	(Nagai *et al.*, 2003)

1.2.1 The role of sponge-associated bacteria in sponge biology

Earlier studies have demonstrated the role of bacteria as a food for the sponges as well as a supplier of fatty acids from phospholipid synthesis (Reiswig, 1975; Schumann-Kindel *et al.*, 1997). From a nutritional perspective, sponges should provide suitable niches for microorganisms compared with the nutrient-poor seawater, particularly in tropical regions. In many sponges the bacteria have been shown to play a role in the lives of their host, either through processing of waste products (Beer and Ilan, 1998) or production of secondary metabolites (Unson *et al.*, 1994). Thus, sponges are characterized by complex microbial communities that give opportunities for interactions that could lead to greater diversity of natural products, and can also complicate microbiological analysis. Several lines of evidence indicated that some sponges obtain a significant portion of their nutrients from the bacterial symbionts, making the symbiosis a true mutualism (Haygood *et al.*, 1999; Hentschel and Steinert, 2001). The question of whether sponge-associated bacteria should be regarded as symbionts has often been debated. Symbiosis generally implies that at least one organism benefits from the other (Hentschel *et al.*, 2003).

The contribution of the bacterial partner to their host has been confirmed in several invertebrate host model systems, the reciprocal benefit from the host to the bacterial symbionts is naturally much more difficult to identify (Steinert *et al.*, 2000). Possible symbiotic functions include the exchange of primary metabolites contributing to nutrition, and the production of secondary metabolites for chemical defense purposes. As an example, the presence of cyanobacteria in sponges suggested the hypothesis of symbiotic microbes in sponges. Many shallow-water sponges with cyanobacteria exhibit mechanisms that allow bacterial contribution to the host nutrition through extracellular lysis and phagocytosis (Wilkinson, 1978b; Wilkinson and Garrone, 1980). Moreover, symbiotic cyanobacteria have been shown to fix nitrogen and provide their hosts with amino acids and also to control the redox potential within sponge tissue via photosynthesis (Wilkinson, 1987; Wilkinson *et al.*, 1999). Different colour forms of sponges are attributed to the presence of different cyanobacteria and their production of different secondary metabolites (Thakur and Müller, 2004). Not only that, ammonia is an end product of the host metabolism, it is likely to be available as a nitrogen source, while carbohydrates and amino acids should be

available as a result of the extensive phagocytosis of the host (Davy *et al.*, 2002). Indeed, bacteria within sponge tissues are clearly metabolically active, as evidenced by bright FISH signals, which serve as indirect indicators of cellular rRNA content. Provided that the bacteria can avoid being digested, the mesohyl should be a stable and nutritionally rich habitat.

1.2.2 Symbiosis between bacteria and sponges

Symbiotic relationships can be defined as mutualistic interactions with positive effects for both partners. The paradigm of sponge-microbial symbiosis has been claimed many times in the literature. The following observations have given support to this hypothesis: (1) sponges are associated with large amounts of bacteria (Fuerst *et al.*, 1999); (2) some bacterial morphotypes appear to be specific to sponges (Sara *et al.*, 1998; Ereskovsky *et al.*, 2005); and (3) some bacterial types are permanently associated with sponges even over large temporal and geographic distances (Hentschel *et al.*, 2002). Possible symbiotic functions that have been proposed in the literature include a source of nutrition by way of intracellular digestion and or by translocation of metabolites (Wilkinson and Garrone, 1980), access to novel, bacteria-specific traits such as autotrophy, nitrogen fixation and nitrification (Wilkinson and Fay, 1979), processing of metabolic waste (Beer and Ilan, 1998), stabilization of sponge skeleton or structural rearrangement of the sponge mesohyl (Wilkinson *et al.*, 1981), and involvement in chemical defense against predators and biofouling (Bakus *et al.*, 1986; Unson *et al.*, 1994; Jayatilake *et al.*, 1996).

The importance of these photosynthetic symbionts for the nutrition of the sponge can be considerable (Althoff *et al.*, 1998). As an example, an experiment was conducted to measure oxygen consumption rates in ten sponge species of the Great Barrier Reef of which nine contained cyanobacterial symbionts (Wilkinson, 1983). Six species showed net photosynthesis, indicating that the primary production by the symbionts was higher than the total respiration of the sponge-symbiont consortium. The sponges will consume a substantial part of the primary production, sometimes by direct ingestion of the symbionts, but mostly in the form of excreted metabolic products such glycogen or glycerol (Wilkinson, 1983). Providing nutrition might not be the only role of photosynthetic

symbionts in sponges. Light shielding has been suggested as an alternative function of cyanobacterial symbionts (Wilkinson, 1983). Furthermore, other study has found that the presence of zooxanthellae in the cells of the boring sponge, *Anthosigmella varians,* not only enhanced the growth rate of the sponge, but also its boring capacity (Hill, 1996). He suggested a direct physiological role of the endosymbionts in the decalcification of the calcium carbonate substratum. Sponge-symbionts may also affect the nitrogen metabolism of their host. Previous study described the presence of nitrogen fixing bacteria in the Indo-Pacific coral reef sponge, *Callyspongia muricina* (Wilkinson *et al.*, 1999). In low nutrient environments uptake of inorganic nitrogen via nitrogen fixation may be essential to sponges that grow on photosynthetically derived carbohydrates.

Another role of symbionts is the production of potentially useful chemical compounds such as antibiotics, antifungal compounds and compounds that prevent predation or fouling (Fenical, 1996; Haygood *et al.*, 1999). A well-described example of this is the production of antimicrobial polybrominated biophenylethers and polychlorinated peptide by *Oscillatoria spongelia*, a cyanobacterial symbiont of the Indo-Pacific sponge, *Dysidea herbacea* (Thakur *et al.*, 2003; Flatt *et al.*, 2005). It was suggested that the brominated biphenylethers are produced to keep the sponge tissue free of other bacteria (Unson *et al.*, 1994). It has been observed that the surface of the sponges is very clean, and several compounds with strong anti-fouling capacities have been isolated from sponges (Miki *et al.*, 1996).

In addition to this direct protection or defense strategy, an indirect protection system has been developed by sponges against infecting bacteria (Thakur *et al.*, 2003). Infection studies have revealed that sponges can also harbor, depending on the environmental conditions, antimicrobially active bacteria on their surface which support epibacterial defense (Fig. 1.4). This suggests that symbionts are important in sponge ecology and allows for the evolution of a more permanent association with co-speciation (Wilkinson, 1983, 1984).

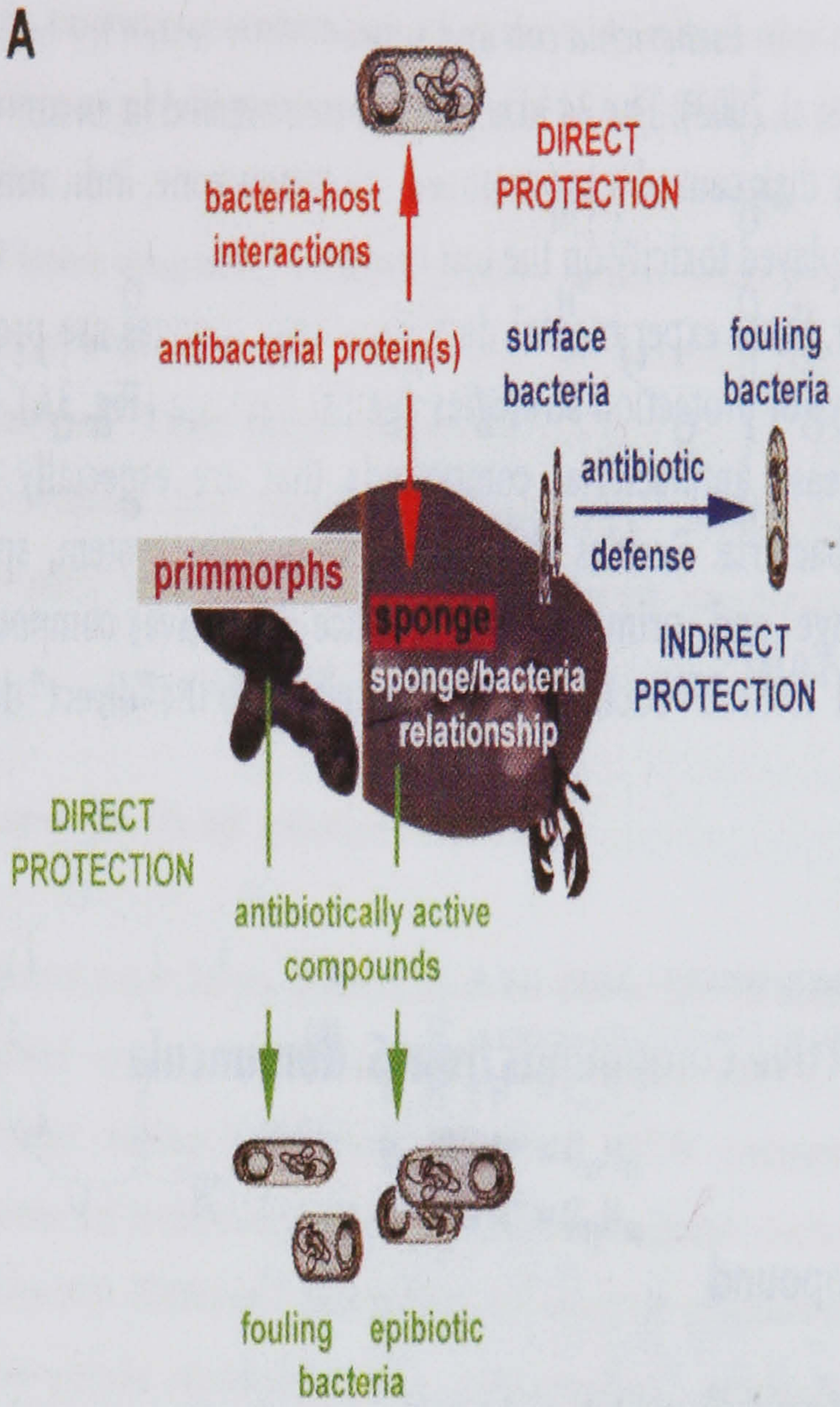


Figure 1.4. Defense systems of sponges with *S. domuncula* as a model. Schematic outline of the strategies of sponges to defend themselves against bacteria. Indirect bacteria-mediated defense: surface-associated bacteria release anti-bacterial compounds that inhibit growth of fouling bacteria. Direct defense: antibiotic active compounds, produced and released by sponge / primmorphs and inhibit growth of both fouling and epibiotic bacteria (Müller *et al.*, 2004)

Ultrastructural studies have demonstrated vertical transmission of bacterial symbionts with sponge larvae. A common technique to detect evidence for co-speciation is the comparison of host and symbiont phylogenies (Casiraghi *et al.*, 2001). If symbionts and host co-evolve, their phylogenies are expected to be congruent (Page, 1994). If, on the other hand, symbionts have been acquired independently many times or if associations are not specific, congruent phylogenies are not expected. In several marine systems, such congruent phylogenetic patterns have been observed (Distel *et al.*, 1994), but records for sponge-symbiont co-evolution are lacking. At present, molecular biological techniques have allowed first insights into the functional symbiotic relationship between bacteria and their host.

1.2.3 Species-specificity of sponge bacteria

Several studies have now been undertaken to show the diversity of bacteria associated with sponges (Hentschel *et al.*, 2003; Imhoff and Stohr, 2003; Hill, 2004). 16S rDNA gene library construction using universal bacterial PCR primers have revealed a common microbial signature in many sponges that is phylogenetically complex yet highly sponge-specific and distinctly different from that of marine plankton. 16S rDNA gene phylotypes affiliated with the phyla *Acidobacteria*, *Chloroflexi*, *Actinobacteria*, *Proteobacteria* (α, γ, δ), *Nitrospira*, *Cyanobacteria*, and *Bacteriodetes,* as well as a cluster of uncertain affiliation now recognized as the *Gemmatimonadetes* were recovered by this approach. In addition, a crenarchaeal lineage was documented in *Axinella mexicana* and other axinellid sponges (Preston *et al.*, 1996; Margot *et al.*, 2002), and a new eubacterial candidate phylum 'Poribacteria' was recently discovered in verongid sponges (Fieseler *et al.*, 2004). The fact that the apparently sponge-specific phylotypes are present below detection levels or may even be absent from seawater, sediment and other environments indicates highly selective conditions for microbial existence within the sponge mesohyl.

As common patterns are beginning to emerge, so are the exceptions. Among three Australian temperate reef sponges investigated (Taylor *et al.*, 2004b), only one, *Cymbastela concentrica* showed convincing evidence for sponge-specific phylotypes. In contrast, the microbial profiles of the co-occuring *Callyspongia* spp. and *Stylinos* spp. were highly

similar to the seawater. Another study performed a molecular diversity analysis of five Antarctic sponges (Webster *et al.*, 2004). The microbial diversity within these sponges was substantially lower than that of seawater; the phylotypes were most closely repeated to previously retrieved sequences from polar (nonsponge) environments, and monophyletic clusters, as describes by others were not evident (Hentschel *et al.*, 2002), Another study addressed the microbial community composition of a freshwater sponge (Gernert *et al.*, 2005). The microbial diversity of *Spongilla lacustris* was comparably small and largely resembled that of the surrounding lake water. A number of studies have demonstrated the diversity of sponge-associated microbial communities by using cultivation-based approaches and revealed that the microbial communities can be quite similar (Friedrich *et al.*, 1999; Bohm *et al.*, 2001; Webster and Hill, 2001; Webb and Maas, 2002). In fact other study found that an α-proteobacterium isolated from two different species of sponge, *Forcepia* sp. and *Discodermia* sp., (Olson *et al.*, 2002) was closely related to the isolate α-proteobacterium, NW001 (Webster and Hill, 2001) from the sponge *Rhopaloeides odorabile,* and MBIC3368 from the sponge *Aplysina* sp. (Friedrich *et al.*, 1999). In addition, at the molecular level, other research has hypothesized that sponges have uniform microbial communities irrespective of sponge species and location (Hentschel *et al.*, 2002). However, this finding is not applied to all sponges. Recent reports indicated substantial variability occurred among microbial communities from the different sponges, *Cymbastela concentrica*, *Callyspongia* sp. and *Stylinos* sp. (Taylor *et al.*, 2004b).

1.3 IDENTIFICATION OF MICROORGANISMS ASSOCIATED WITH SPONGES

1.3.1 Microscope observations

Bacteria-sponge associations have been studied using three major methodological approaches: (1) classical microscope observation, (2) pure culture isolates, and (3) genetic analyses of sponge-associated bacteria. The first information that stressed the important role of bacteria in sponges came from microscope studies demonstrating the presence of large numbers of bacteria within marine sponges (Vacelet and Donadey, 1977; Wilkinson, 1978c). These investigations gave insights into the distribution and localization of bacteria

within the sponges. Large numbers of bacteria were found not only inside the vacuoles of archaeocytes (Simpson, 1984), but also in the nucleus of sponge cells (Flowers *et al.*, 1998; Friedrich *et al.*, 1999), and even outside the sponge cells in the mesohyl, which is located between the cell layers that line the canal system and the choanocytes.

On the basis of morphological studies, attempts were made to distinguish between sponge-associated and seawater bacteria under the microscope (Wilkinson, 1978a, 1978b, 1978c). Three broad categories of bacteria associated with sponges were recognized: (1) bacteria that were similar to those of ambient seawater and not specific to the sponge; (2) small numbers of intracellular bacteria that were considered to be specific to the sponge; and (3) large numbers within the mesohyl that appeared to be specific. As an example, in *Aplysina cavernicola* five dominant bacterial morphotypes were identified that were regarded as being specifically associated with the mesohyl (Vacelet and Donadey, 1977).

In addition to information on localization, cell numbers, form and size of the cells, some outstanding properties can be recognized by electron microscopic studies, particularly the type of cell wall (Gram-positive or Gram-negative) and internal membrane structures that characterize quite a few specific groups of bacteria. Their internal membrane systems allowed the presence of cyanobacteria within sponges to be recognized quite easily (Hinde et al., 1994). Because morphological characteristics, in general, are very poor properties for bacterial classification, accurate identification of sponge-associated bacteria could not be achieved by microscope observations.

1.3.2 Cultivation of sponge-associated bacteria

Classical microbial techniques and cultivation approaches have been used in a number of studies to analyze the diversity of bacteria within sponges. Although bacterial cultivation is a prerequisite to study the physiological basis of possible interactions between bacteria and sponges, its contribution to analyze bacterial diversity is limited by the cultivation success (Santavy *et al.*, 1990; Webster and Hill, 2001). Cultivation of bacteria is always highly selective due to the choice of media and culture conditions which usually allows only a small fraction of the bacteria present within a sponge to grow and in consequence to be

isolated. Special skills are required to include those bacteria that are of interest. No selective cultivation is currently available to distinguish bacteria specifically associated with sponges from others, including those bacteria that serve as food particles. Therefore, cultivation approaches can only be part of a more general concept to define the diversity of bacteria associated with sponges and to identify the function they play within these complex associations.

Selective culture conditions have allowed bacterial isolates with special physiological properties to be obtained in many studies. These conditions helped categorize many bacterial groups including aerobic chemoheterotrophic bacteria (Wilkinson *et al.*, 1981), nitrogen-fixing bacteria (Shieh and Lin, 1994), methane-oxidising bacteria (Vacelet *et al.*, 1996) and phototrophic cyanobacteria (Imhoff and Trüper, 1976). More recently novel actinobacteria were cultivated from several marine sponges (Kim *et al.*, 2005; Montalvo *et al.*, 2005). In a number of studies bacteria isolated from sponge material were identified and compared with those obtained from ambient seawater that was treated in a similar or identical way (Webster and Hill, 2001; Müller *et al.*, 2004). However, caution is indicated with all the conclusions on a specific association of bacteria with sponges that result from culture experiments and identification of isolated bacteria, because the high selectivity of culture media would allow similar or identical bacteria to be specifically obtained from different sources and at different times. In addition to more general limitations of the cultural approach, unfortunately most of these reports presented limited taxonomic information, because often the bacteria were assigned to genera, but not identified to the species level.

1.3.3 Use of molecular techniques to identify bacteria associated with sponges

The application of molecular techniques to identify sponge-associated bacteria and to analyse their diversity opened new horizons in the analysis of associations between sponge and bacteria. These techniques provide culture-independent tools for analysis of bacterial community structure and allow verification of the localization of identified bacteria within the sponge (Hentschel *et al.*, 2003; Hill, 2004; Hentschel *et al.*, 2006). This is one important prerequisite to prove their presumed symbiotic role. Therefore, molecular

techniques that enable identification and localization within the sponge are highly promising to enlighten the specificity of bacteria-sponge interactions, especially if they are combined with cultural approaches and microscopic investigations. Different genetic approaches have been applied to verify the diversity and identity of sponge-associated bacteria. Several lines of study have applied sponge-derived DNA approaches (Hentschel *et al.*, 2002; Thiel and Imhoff, 2003). Sponge-derived DNA was amplified, cloned and clone sequences of amplified 16S rDNA segments were obtained. By this technique species–specific sequences can be obtained and, under consideration of the limited quantitative information from genetic amplification experiments, also information on the relative abundance of individual sequences and of sequence clusters.

Other approaches such as fluorescent gene probes that specifically recognize prokaryotic groups or even species have been applied to sponge sections. This technique, FISH, yields information on the abundance of the groups recognized by the applied gene probes and in addition on their location within the sponge. Other novel approaches using DGGE have resulted in a remarkable array of new discoveries on marine microbial communities in sponges (Thoms *et al.*, 2003; Taylor *et al.*, 2004b). DGGE analysis is a particularly useful application for the characterization of sponge-associated microbial communities as it not only provides insights into the overall complexity of the microbial community, but also allows the monitoring of changes in community composition of individual sponges over time. Using DGGE in combination with other techniques, studies have shown that the microbial community of *Aplysina aerophoba* was resistant to experimental perturbations, such as exposure to starvation and/ or to antibiotics over a time course of 11 days (Friedrich *et al.*, 2001). Studies on the diversity of sponge-associated eubacteria, which include different genetic approaches, have been made recently with the sponge *Rhopaloeides odorabile* (Webster and Hill, 2001; Webster *et al.*, 2001a) with *A. aerophoba* and *A. cavernicola* (Friedrich *et al.*, 1999; Friedrich *et al.*, 2001; Hentschel *et al.*, 2001), and with *H. panicea* (Althoff *et al.*, 1998).

Bacterial communities associated with *Rhopaloeides odorabile* were quite diverse as indicated by relatively low duplication in a clone library of 70 clones. The major bacterial groups identified by analysis of a 16S rDNA clone library were actinobacteria (30 % of the

clones), γ-proteobacteria (41 % of clones) and low G+C Gram-positive bacteria. β-proteobacteria, *Cytophaga* or *Flavobacterium*, green sulphur bacteria, green nonsulphur bacteria, Planctomycetes and others without known relatives were also found (Webster *et al.*, 2001b). The FISH technique using several group-specific probes confirmed these results. Application of these probes revealed the spatial location of bacterial groups within the sponge and indicated that γ-proteobacteria were especially dominant around the choanocyte chambers (Webster *et al.*, 2001c).

1.4 THE DIVERSITY OF NATURAL PRODUCTS FROM SPONGES

Most studies have been directed towards chemical studies of marine invertebrates. The MarinLit database (Marinlit, 2001) shows that marine invertebrates are an important source of new biomedical compounds. These compounds range from derivatives of amino acids and nucleotides to macrolides, porphyrins, terpinoids, and sterols with diverse bioactives (Thakur and Müller, 2004). Sponges have more bioactive molecules than any other marine invertebrate, consistent with their need for chemical defenses as sessile animals, and in some cases such molecules are thought to be from symbiotic bacteria (Haygood *et al.*, 1999). However, the exact origin of these compounds is still under investigation (Zheng *et al.*, 2000; Lee *et al.*, 2001). Isolation of new marine bacteria from marine sponges can contribute to our knowledge of such sponge bioactive compounds and their origins. Furthermore, the production of biologically active secondary metabolites may act as repellents against predators and also to manage the control of the sponge-associated bacteria and microfauna (Faulkner *et al.*, 2000).

1.4.1 Production of secondary metabolites

1.4.1.1 Secondary metabolites produced by sponges

A number of studies have shown that the origin of secondary metabolites may come from sponge cells as well as the symbionts (Table 1.2). For example, localization of the toxin Latrunculin B has been revealed in the sponge cells, *Negombata magnifica,* but not in symbiotic bacteria (Gillor *et al.*, 2000). The same phenomenon was observed for

sphingosine derivative in the sponge cells of *Haliclona vansoesti* (Richelle-Maurer *et al.*, 2001). However, recent evidence is in favour of both origin of sponge, *S. domuncula* and primmorph system in the production of okadaic acid (Wiens *et al.*, 2003). *S. domuncula* uses okadaic acid as defense against foreign eukaryotic organisms while at the same time it possesses a relative resistance against this compound.

It has been demonstrated that the spectrum of bioactive compounds within different sponge taxa varies. As an example, the brominated metabolites derived from tyrosine are characteristic for the order Verongia (Bergquist *et al.*, 1998) or the isonitriles for the order Halichondria (van Soest, 1991). Consequently, marker compounds have been described for different sponge groups and successfully used to support taxonomy (van Soest and Braekman, 1999).

1.4.1.2 Metabolites isolated from sponges but actually produced by associated bacteria

Ample evidence has been presented suggesting associated bacteria are the main source for the production of the bioactive compounds isolated from the host (Althoff *et al.*, 1998; de Rosa *et al.*, 2001; Lang *et al.*, 2004; Ramm *et al.*, 2004). However, these results do not rule out the possibility that substances might be transported between cell types via export or sequestration mechanisms (Piel *et al.*, 2005). The existence of producing symbionts could have far reaching consequences for the development of sustainable, fermentation-based sources of invertebrate-derived drug candidates, almost all of which are currently inaccessible in large amounts. Several studies have tried to pinpoint the producers by using cultivation, cell separation, immunolocalization, or *in situ* hybridization approaches (Faulkner *et al.*, 2000; Gillor *et al.*, 2000; Schmidt *et al.*, 2000; Davidson *et al.*, 2001). However, as none of these techniques have provided conclusive results, the true origin of the compounds still remain an enigma (Piel *et al.*, 2004). Recent studies of the culturable sponge microbial communities have resulted in the identification of new natural compounds with diverse biological activities (Thiel and Imhoff, 2003; Piel, 2004).

Sponges have been considered as 'microbiological fermentors' for new natural products with potential biotechnological applications (Thakur and Müller, 2004; Hentschel *et al.*, 2006). Furthermore, metagenomic approaches have revealed novel chemistry of the uncultivated symbionts in the marine sponges *Theonella swinhoei* and *Discodermia dissolute* and have opened a new avenue for a better understanding of the valuable natural compounds from symbiotic bacteria in sponges (Piel, 2004; Piel *et al.*, 2005; Schirmer *et al.*, 2005). As an example, studies indicate yield of bioactive glucosylmannosyl glycerolipid from cultivation of a marine *Microbacterium* isolated from *H. panicea* which was shown to inhibit growth of tumor cell lines (Lang *et al.*, 2004). Another example of the same activity is from *Bacillus pumilus* strain AAS3, isolated from the Mediterranean sponge *Acanthella acuta*, which produced a diglucosyl-glycerolipid (Ramm *et al.*, 2004).

The isolated bacteria from *S. domuncula* showed species-level similarity to the α–proteobacterium MB1C3368 (Thakur *et al.*, 2003), which displayed antimicrobial activities. Interestingly, isolates of the same bacteria were also recovered from the Mediterranean sponge, *Aplysina aerophoba* (Hentschel *et al.*, 2002) as well as the Australian sponge, *Rhopaloeides odorabile* (Webster *et al.*, 2001), which displayed similar activities against various Gram-positive and Gram-negative bacteria. A thorough understanding of the microbial communities associated with marine sponges may be important in terms of natural products discovery, since sponges may provide many novel isolates for natural products screening programs.

1.4.2 Secondary metabolites with potential use for medicine

Bacteria associated with sponges have developed unique metabolites and physiological capabilities that not only ensure survival in extreme habitats, but also offer the potential for the production of metabolites not observed in terrestrial microorganisms (Garson, 2001). The biological effects of new metabolites from sponges have been reported in many scientific papers. Sponges have the potential to provide future drugs against important diseases, such as cancer, a range of viral diseases, malaria, and inflammations (Schwartsmann, 2000; Proksch *et al.*, 2002; Sipkema *et al.*, 2005).

Table 1.2 Selected secondary metabolites isolated from sponges

Secondary Metabolite	Compound Activity	Sponge species	Source	References
Latrunculin B	Defense toxin	*Negombata magnifica*	Sponge cells	(Gillor *et al.*, 2000)
Agelasine F Antituberculosis	Diterpenoid	*Agelas* sp.	Sponge cells	(Mangalindan *et al.*, 2000)
Manzamine A Antifouling	Alkaloids	*Haliclona* sp.	Sponge cells	(Sera *et al.*, 2003)
Arenoclerins A-C Antibacteria	Alkaloids	*Arenoslera brasiliensis*	Sponge cells	(Torres *et al.*, 2002)
Cycloperoxides Antimalaria	Cycloperoxide	*Plakortis simplex*	Sponge cells	(Fattorusso *et al.*, 2002)
Okadaic acid Immune response	Polyether fatty acid derivative	*S. domuncula*	Sponge cells	(Wiens *et al.*, 2003)

Although the molecular mode of action of most metabolites is still unclear, for a substantial number of compounds the mechanisms by which they interfere with the pathogenesis of a wide range of diseases have been reported. Many new molecules with antibiotic properties are discovered every year, but in marine sponges their ubiquity is remarkable (Fenical and Jensen, 2000; Matsunaga *et al.*, 2001). The added value of some new sponge-derived antibiotics was shown by the inhibitory effect of arenosclerins A-C from *Arenosclera brasiliensis* on 12 antibiotic resistant bacteria isolated from a hospital (Torres *et al.*, 2002). The isolation of antimicrobial metabolites from bacteria collected from sponges suggested that these bacteria may play a role in a defense mechanism of these invertebrates (Kasanah and Hamann, 2004). In the continuing effort by the marine natural products community, many antimicrobial agents have been identified. As an example, cribrostatins were isolated from a blue sponge, *Cribrochaliana* sp., and showed potent antimicrobial activities. Cribrostatin 3 has potent inhibitory activity against *Neisseria gonorrheae*, with an MIC of 0.09 μg/ml (Pettit *et al.*, 2000). Other examples of antimicrobial metabolites [2,4,4'-trichloro-2'-hydroxydiphenyl ether and acyl-1-(acyl-6'-mannobiosyl)-3-glycerol] were obtained from a bacterium identified as the ubiquitous Gram-positive bacterium *Micrococcus luteus,* which was isolated from the sponge *Xestospongia* sp. (Bultel-Poncé *et al.*, 1998).

Sponges have been proven to be an interesting source of anti-inflammatory compounds. Monoalide, one of the first sesterterpenoids to be isolated from a marine sponge, *Luffariella varialilis*, was found to be an antibiotic and an analgesic (Mayer *et al.*, 1998). In addition, its anti-inflammatory properties have been studied extensively. The anti-inflammatory action is based on the irreversible inhibition of the release of arachidonic acid from membrane phospholipids by preventing the enzyme phospholipase A2 from binding to the membranes (Glaser *et al.*, 1989).

The anti-inflammatory sponge products are selective inhibitors of specific enzymes of a range of diseases, like psoriasis or rheumatic arthritis. The currently used non-steroidal anti-inflammatory drugs often fail to control the disease and present important side effects, such as risk of gastrointestinal bleeding and renal complications (de Rosa, 2000). These are caused by unselective inhibition of cyclooxygenases, some of which are also involved in

the promotion of the production of the natural mucus that protects the gastrointestinal tract (Bjarnason *et al.*, 1993). Many non-specific cell growth inhibitors have been discovered in sponges. They are valuable for treating cancer under certain conditions, but they also affect the division of healthy cells. Therefore, their applications are limited, depending on their specific characteristics. The cytoskeleton is an interesting target for cancer therapy, as the microtubules and microfilaments are involved in cellular organization during cell division. A number of adociasulphates (triterpenoid hydroquinones) from a *Haliclona* sp. were the first inhibitors of the kinesin motor protein to be discovered. These toxins are believed to inhibit the protein by binding to the microtubule binding site, 'locking up' the protein's motor function, and thereby blocking cell division (Blackburn *et al.*, 1999). In addition to these triterpenoid hydroquinones, a number of potent microtubule-interfering compounds have been discovered in marine sponges, such as halichondrin B (Bai *et al.*, 1991), spongistatin (Bai *et al.*, 1993), laulimalide (Mooberry *et al.*, 1999), peloruside A (Hood *et al.*, 2002), and dictyostatin (Isbrucker *et al.*, 2003).

In addition, many more compounds that displayed growth inhibition activity of tumor cell lines have been isolated from sponges, although their exact effects are still unclear. Discorhabdin D (Perry *et al.*, 1988), chondropsin A and B (Cantrell *et al.*, 2000), haligramides A and B (Rashid *et al.*, 2000), and glaciasterols A and B (Pika *et al.*, 1992) are only new examples of these molecules. The potency of sponge-derived medicines lies in the fact that each of these metabolites and their derivatives has its own specific dose-related inhibitory effect, efficacy, and potential side effects that determine its suitability for medical use. In addition, the skeleton or active core of these molecules may be used as a vehicle to develop derivatives with their own specific efficacy and side effects.

1.5 OXIDATIVE STRESSES

Oxidative stress can be functionally defined as an excess of prooxidants in the cell (Storz and Imlay, 1999). Active oxygen intermediate molecules such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO), have been shown to cause damage to proteins, nucleic acids, and cell membranes. Oxygen by itself is unable to cause any damage to the cell; however, during the cellular processes (metabolic pathway) O_2^- is

partially reduced to water, leading to the formation of reactive oxygen species. These intermediates have a high oxidizing potential and thus are responsible for cellular oxygen toxicity (Farr and Kogoma, 1991). Oxygen toxicity results when the degree of oxidative stress exceeds the capacity of the cell defense systems. Oxidative stress is strongly implicated in a number of diseases such as rheumatoid arthritis, aging and atherosclerosis (Halliwell and Gutteridge, 1990). Virtually all aerobic organisms have evolved complex defense and repair mechanisms to mitigate the damaging effects of active oxygen (McCord and Fridovich, 1988).

1.5.1 Oxidative stress in E. coli

1.5.1.1 Oxidative stress damage at the metabolic level

Oxidative stress is unavoidable by product of the aerobic lifestyle, because O_2 and H_2O_2 are formed whenever molecular oxygen chemically oxidizes electron carriers. Reduced flavoprotein in particular has been implicated in this process in *E. coli*. In exponentially growing *E. coli*, both O_2 and H_2O_2 are generated by the autoxidation of components of the respiratory chain (Aldea *et al.*, 1989). The flavin of NADH dehydrogenase II is the primary site of electron transfer to oxygen in the aerobic respiratory chain; contrary to expectation, little or no O_2^- or H_2O_2 are formed by quinine oxidation or during oxygen reduction at the cytochrome oxidases (Ames, 1983). Fumarate reductase, a terminal oxidase that is induced during anaerobic growth, reacts very rapidly with oxygen and may confer particular oxidative stress when cells transit from anaerobic to aerobic environments (Amstad and Cerutti, 1990). The expression of both NADH dehydrogenase II and fumarate reductase is regulated in *E. coli* and the enzymes are not present in all bacteria; thus, the amount of endogenous oxidative stress will be affected by growth circumstance and will vary from organism to organism. Aerobic *E. coli* synthesizes enough superoxide dismutase to maintain the steady-state O_2^- from these endogenous sources at about 10^{-10} M. This O_2^- is tolerable; nearly half what is necessary to diminish the activities of vulnerable enzymes and inhibit cell growth (Storz and Imlay, 1999). Steady state H_2O_2 concentrations are higher (10^{-7} to 10^{-6}) but still beneath the H_2O_2 toxicity threshold (10^{-5}).

1.5.1.2 Oxidative stress damage at molecular level

At the molecular level O_2^-, HO and H_2O_2 can react with cellular targets, such as proteins and DNA. O_2^- has a moderate oxidizing potential and can attack compounds such as polyphenols, ascorbate and catecholamines (Farr and Kogoma, 1991). H_2O_2 can directly oxidize protein cysteinyl residue, thus inactivating enzymes (Storz and Imlay, 1999). It can also react with cations, such Fe^{2+} and Cu^{2+}, and give rise to $HO^{\cdot}$, through the Fenton reaction (Storz and Imlay, 1999). H_2O_2 also react with adventitious Fe^{2+} to form $HO^{\cdot}$, a powerful oxidant that reacts at diffusion-limited rates with most biomolecules. $HO^{\cdot}$ can cause strand breaks and a wide spectrum of base modifications in DNA (Storz and Imlay, 1999). Another type of damage is the peroxidation of membrane lipids and membrane protein alterations, affecting cell permeability and osmoregulation (Farr and Kogoma, 1991).

1.5.2 Oxidative stress resistance mechanisms in E. coli

Accordingly, *E. coli* has developed antioxidant mechanisms to overcome damage caused by active oxygen species. Experimental data and genomic analyses indicate that *E. coli*, similar to *Lactococcus lactis* and *Bacillus subtilis*, is equipped with general and specific stress response mechanisms (Duwat, 1999). Several genes that participate in these mechanisms have been identified and the respective encoded proteins have been shown to contribute to oxidative stress resistance. Moreover, induction of some of these genes is growth phase-dependent, and their products confer multi-stress resistance (Farr and Kogoma, 1991). To protect against the damage caused by oxidative stress, cells possess a number of antioxidant enzymes and repair activities, most of which are expressed at low levels during normal growth. In response to elevated concentrations of O_2^- and H_2O_2, the expression of many antioxidant proteins is induced. Among the O_2^- inducible activities are regulated by SoxRs transcription factors are manganese superoxide dismutase (*sod*A), the DNA repair enzyme endonuclease IV (*nfo*), and O_2^- resistant isozymes of fumarase (*fum*C) and aconitase (*acn*A) (Jamieson and Storz, 1997). SoxRS activation can lead to increased levels of glucose-6-phosphate dehydrogenase (*zwf*), which increases the reducing power of

the cell, and elevated levels of the Fur repressor (*fur*), which may decrease iron uptake and therefore diminish the formation of OH (Miller and Sulavik, 1996; Jamieson and Storz, 1997; Aono *et al.*, 1998). The *tol*C-encoded outer membrane protein, the *acrA*B-encoded drug efflux pump, and the MicF regulatory RNA, which represses the expression of the outer membrane porin, all are likely exclude redox-active compounds that lead to increased O_2^- levels (Miller and Sulavik, 1996; Jamieson and Storz, 1997).

Two enzymes that clearly protect against O_2^- damage, but are not regulated by SoxRS, are cytosolic iron superoxide dismutase (*sod*B) and the periplasmic copper-zinc superoxide dismutase (*sod*C) (Fridovich, 1995, , 1998). It is also conceivable that *a* homologue of NifS, which was identified as a protein that provides sulfur for the Fe-S cluster assembly in *Azotobacter vinelandii*, might protect against O_2^- stress, but neither the expression nor the physiological role of this gene has been studied extensively in *E. coli* (Flint, 1996). The expression of many of the H_2O_2–inducible activities is regulated by the OxyR transcription factor, including hydroperoxidase I (catalase, katG), the two subunits of an alkyl hydroperoxidase reductase (ahpCF), glutaredoxin 1 *(grx*A*),* glutathione reductase (*gor*A*)*, and the Fur repressor (fur) (Jamieson and Storz, 1997; Tao, 1997; Zheng *et al.*, 1998). The phenotypes of mutations in the OxyR-regulated genes dps and OxyS indicate that the non-specific DNA-binding protein Dps and the OxyS regulatory RNA protect against mutagenesis. In addition to protecting against O_2^- and H_2O_2 induced damage, the SoxRS regulon provides resistance to many different drugs as well as organic solvents and reactive nitrogen species (Martinez and Kolter, 1997), and OxyR-regulated activities have been found to confer resistance to HOCI, organic solvents, and reactive nitrogen species (Grant *et al.*, 1998). For example, the OxyR-regulated AhpC protein protects cells against reactive nitrogen intermediates, an AhpC activity that is independent of the AhpF subunit required for protection against peroxidases.

1.6 INTERCELLULAR SIGNALLING AND COMMUNICATION OF BACTERIA

In my study some observations may suggest intercellular communication involvement in the production of antimicrobial compounds. Some cell-cell signaling models have been

well established for intercellular communication. Although there is no strategy for the discovery of novel antimicrobial compounds based on induction mechanisms in microorganisms such as quorum sensing, there has been evidence suggesting that quorum sensing is related to the production of antimicrobial compounds by various genera (Kleerebezem and Quadri, 2001).

1.6.1 Quorum sensing

Quorum sensing is a regulatory mechanism by which bacteria control gene expression in response to population density (Fuqua *et al.*, 1994). In Gram-negative bacteria quorum sensing involves acyl-homoserine lactone (AHL) signal molecules, produced by members of the LuxR family of transcriptional activators, which mediate the response to local concentrations of AHL (Hastings and Greenberg, 1999). The first AHL, 3-oxo-hexanoyl-AHL (3-oxo-C6-AHL) and the luxI and luxR genes were first identified in the marine bioluminescent bacterium, *Vibrio fischeri* (Engebrecht and Silverman, 1984). Light emission by these bacteria is tightly correlated with the cell population density. Cells produce and release an AHL pheromone into the extracellular environment. When cell density is low, the concentration of pheromone in the surrounding environment of bacteria is low, and most cells do not emit light. With increase in cell population density, the released AHL accumulates in the niche where bacteria are grown. When a minimal threshold stimulatory concentration is achieved, a signaling cascade is elicited in *V. fischeri* cells and culminates in the emission of light (Engebrecht and Silverman, 1984; Fuqua *et al.*, 1994). At present, LuxI/LuxR quorum-sensing systems have been identified in over 25 species of Gram-negative bacteria from diverse habitats, including both marine and terrestrial bacteria and several pathogens of plants and animals (Swift *et al.*, 1999).

Quorum sensing controls various different activities in these different bacteria, including luminescence, the production of extracellular enzymes, plasmid transfer, antibiotic synthesis, and the biofilm formation (Fuqua *et al.*, 1994; Swift *et al.*, 1999). In some species, quorum sensing coordinates the expression of several unlinked genetic loci. For example, in the opportunistic human pathogen, *Pseudomonas aeruginosa*, two LuxI/LuxR homologue pairs, LasI/LasR and Rh1I/Rh1R, regulate more than 40 genes via a complex

network (Whiteley *et al.*, 1999). The coordinated production of multiple proteins of diverse function suggests that quorum sensing is an adaptational response to conditions of high population density, such as those encounted in association with plant and animal hosts (Swift *et al.*, 1999). Both Gram-positive and Gram-negative bacteria use quorum sensing as a communication approach to regulate a diverse array of physiological activities, including symbiosis, virulence, competence, conjugation, motility sporulation, antibiotic production and biofilm formation. This phenomenon is mediated by extracellular chemicals named pheromones or autoinducers that act as signal molecules.

1.6.2 Quorum sensing in Gram-negative bacteria

AHL, as a signal molecule mediates quorum sensing in Gram-negative bacteria. AHL molecules comprise a homoserine lactone moiety which is derived from S-adenosylmethionine, and it is linked to an acyl side chain which is derived from an intermediate of fatty acid biosynthesis. Common variations of the N-acyl side chain structure, including chain length and the nature of substituent (usually oxo-) at the C3 position, determine the biological properties of the AHL within a given population (Swift *et al.*, 1999; Miller and Bassler, 2001). One of the typical examples of quorum sensing in Gram-negative bacteria is the regulation of bioluminescence in the symbiotic marine bacterium *Vibrio fischeri* (Fuqua *et al.*, 1994). When free living and at low cell density, cultures of *V. fischeri* appear dark or dim; however, when cells reach a critical concentration the population emits blue-green light. It has been demonstrated that quorum sensing is a ubiquitous phenomenon among bacteria which allow these signal-celled prokaryotic organisms to be engaged in multicellular behaviour macroscopically.

1.6.3 Quorum sensing in Gram-positive bacteria

Like Gram-negative bacteria, quorum sensing also occurs in Gram-positive bacterial species. Although the fundamental purpose of quorum sensing in Gram-negative and Gram-positive bacteria is identical, i.e., the density-dependent expression of target genes via the secretion and detection of an autoinducer signalling molecule, the signalling molecules, mechanism of their synthesis and the secretion and the detection apparatus used by Gram-positive bacteria are not similar to those of Gram-negative bacteria. Gram-positive quorum

sensing bacteria use a secreted peptide as an autoinducer. Typically, the peptide signal molecule is secreted by a dedicated ATP-binding cassette (ABC) transporter (Kleerebezem *et al.*, 1997). Two-component systems consist of a family of homologous proteins that exist in a wide variety of both Gram-negative and Gram-positive bacteria. These systems enable bacteria to adapt to alterations in a wide variety of environmental conditions. Two-component systems relay sensory information by phosphorylation/dephosphorylation cascades. The two components are a membrane-bound response regulator protein, which following phosphotransfer from a cognate sensor kinase, typically controls transcription of downstream target genes (Storz and Imlay, 1999). Gram-positive quorum sensing bacteria use two-component systems to detect and respond to the accumulation of a threshold concentration of a peptide auto-inducer. Several Gram-positive quorum-sensing regulatory systems and the targets they control are described in the *Streptococcus pneumoniae* Competence System, The *Bacillus subtilis* Competence System, and The *Staphyloccocus aureus* Agr System (Kleerebezem *et al.*, 1997).

1.7 STRATEGIES TO PRODUCE NOVEL SECONDARY METABOLITES

1.7.1 Exploration of interesting genes from uncultured bacteria

Acknowledging that an estimated 99% of sponge-associated microorganisms are not amenable to cultivation, the development of novel, cultivation-independent techniques is of high interest (Fuhrman *et al.*, 1993; Hugenholtz *et al.*, 1998; Rondon *et al.*, 1999). Attempts to improve the recovery of microorganisms by manipulating growth media have met with limitation success (Kell *et al.*, 1998). The used of probes based on 5S, 16S and 23S rDNA, together with the isolation, separation and sequencing of DNA, has led to the establishment of new phylogenetic relationships (Hengstmann *et al.*, 1999). This involves the extraction of high molecular weight DNA from the entire microbial community and cloning the resultant pool, called the 'metagenome' into suitable vectors. The BAC cloning vectors have been designed to hold several hundreds kb in size. These BAC or formid vectors are then propagated in surrogate host strains, such as *E. coli,* or specialized over-expression strains. With the generation of large libraries consisting of tens of thousands of clones, the genetic complexity of the original microbial community is maintained. These

metagenomic libraries are then subcloned. Alternatively, the libraries can be screened for genes or operons of interest using PCR primers via Southern hybridization. Because the cloned insert holds large regions of DNA, this molecular method holds promise for heterologous expression of operons encoding for secondary metabolites which have been traditionally difficult to clone because of their large size. With the construction of metagenomic libraries, functional enzymes, ribosomal operons and antibiotics and pigments have been recovered from environmental microbial communities whose large uncultured fraction would have otherwise been inaccessible (Seow *et al.*, 1997; Henne *et al.*, 2000; MacNeil *et al.*, 2000; Gillespie *et al.*, 2002).

Discovery of 16S rDNA sequences that are distinct from currently known microorganisms has radically altered the perception of microbial diversity (Borneman and Triplett, 1997; Smith *et al.*, 2001). Ribosomal RNA analysis suggests that uncultivated organisms are found in nearly every prokaryotic group and several divisions have not known cultivable representatives (Barer and Harwood, 1999). More 16S rDNA libraries should be constructed from sponges to provide an understanding of to what extent the observed patterns can be generalized. Microbial community profiles should be correlated with physical, chemical, taxonomic and ecological parameters. It is likely that the sponge morphology has an effect on microbial community composition. Ecological factors will be relevant for those microorganisms that are regionally limited. Chemical factors will be important if the microbes are responsible for their synthesis. Cultivation of selected, sponge-specific microorganisms should also be performed. By using DGGE enrichments can be monitored, and by using specific FISH probes microbial cells can be phylogenetically identified (Preston *et al.*, 1996; Friedrich *et al.*, 2001).

1.7.2 Cultivation method for culturable bacteria

A majority of microorganisms from the environment exhibit poor culturability in the laboratory using common nutrient media. The available data indicate that through they were metabolically active, some of these cells are either injured or unculturable (Kell *et al.*, 1998). Special skills are required to include those bacteria that are of interest. No selective cultivation method is available to distinguish bacteria specifically associated with sponges

from others, including those bacteria that serve as food particles. At the same time, a great number of microorganisms undergo a dormant state of low metabolic activity and are unable to divide or to form a colony in most commonly used laboratory conditions (Kell and Young, 2000). However, under certain suitable conditions these microorganisms can begin to grow again after a preceeding resuscitation phase. One important cultivation strategy for better recovery of bacterial growth is the provision of a stimulated natural environment. For example, many studies have focused on the culturability of the bacteria in seawater samples collected far from shore. It has been found that bacteria collected from this oligotrophic marine ecosystem have exhibited very good recoverability in low-nutrient media (Connon and Giovannoni, 2002). These bacteria have evolved survival strategies, including responses to starvation, and may reduce their ability to form colonies on nutrient-rich agar (Nystrom *et al.*, 1992). Previous studies have shown that use of a designated diffusion chamber with chemical components added as their natural environment enhanced the growth of uncultivated microorganisms (Kaeberlein *et al.*, 2002).

1.8 AIMS OF THE STUDY

Predatory-prey relationships have been well studied between sponges and bacteria (Bakus *et al.*, 1986; Althoff *et al.*, 1998; Demain, 1998). However, the predator-prey relationship between bacteria and sponges may obscures the possible symbiotic relationship (Faulkner *et al.*, 2000). It has been reported by others that sponge-associated bacteria are involved in the production of antimicrobial compounds (Burja *et al.*, 1999; Faulkner *et al.*, 2000; Schmidt *et al.*, 2000). Nevertheless, these are only a number of very limited studies have clear indications for these phenomena (Schmidt *et al.*, 2000; Bringmann *et al.*, 2003). The overall aims were undertaken as two sub-topics: the first investigated the diversity of sponge-associated bacteria by using culture-dependent and culture-independent methods. The second was to characterize the chemical structure of the antimicrobial compounds from sponge-associated bacteria using large scale-up of culture. The aims of my study are summarized as follows:

1. To isolate and characterize sponge-associated marine bacteria.
2. To investigate the diversity of the total bacterial community within sponges

CHAPTER 2 MATERIALS AND METHOD

CHAPTER 2 MATERIALS AND METHODS

2.1 RECOVERY OF MARINE BACTERIA FROM SPONGES

2.1.1 Collection of sponge samples

Sponges belonging to nine species (*Halichondria panicea*, *H. bowerbanki*, *Suberites domuncula*, *S. carnosus*, *Dysidea avara*, *Chondrosia reniformis, Axinella polypoides, Clione celata,* and *Pachymatisma johnstonia)* were collected from various localities along Loch Creran, Argyll, Scotland. Three sampling sites were involved; site 1 was 56deg. 32.30`N 5 deg 23.88`W from 12m of water and site 2, 'Creran Seamount' was 56deg 31.24`N 5 deg 21.51`W from 16.7m of water. (Figure 2.1). Site 3 was an intertidal area along the Loch Creran. The samples were hand-picked from the intertidal and subtidal regions, while from greater depths (10-20 met) by scuba diving. Sponge specimens (Figure 2.2) were retained in seawater before transporting to the laboratory prior to being preserved in –80 ^{0}C and /or placement in 80% ethanol. Collection occurred throughout 2003 –2005. Seawater samples (10 L) were collected in spring and summer from the waters around the sample habitat and vacuum filtered through 0.45 μm membrane filter and frozen at –80 ^{0}C (Somerville *et al.*, 1989). The samples were cleaned several times with natural seawater and sterile (121 ^{0}C, 15 min) distilled water to avoid contamination from other bacteria from marine environments before microbiological examination (Thoms *et al.*, 2004). Centrifugation was subsequently carried out for all the samples to avoid loosely attached microorganisms.

2.1.2 Isolation of marine bacteria

Sponge samples were individually cut into small pieces, smashed and placed into 5ml volumes of autoclaved (121 ^{0}C, 15 min) seawater and vortexed for ~ 2 min in order to remove any loosely attached microorganisms. The supernatant was serially diluted (10 ^{1}- 10 ^{6}), and an aliquot of 100 μl of each dilution was spread onto three replicate plates of each of the following media; Marine Agar 2212E (MA; Difco, Detroit), Nutrient Agar (NA; Difco),

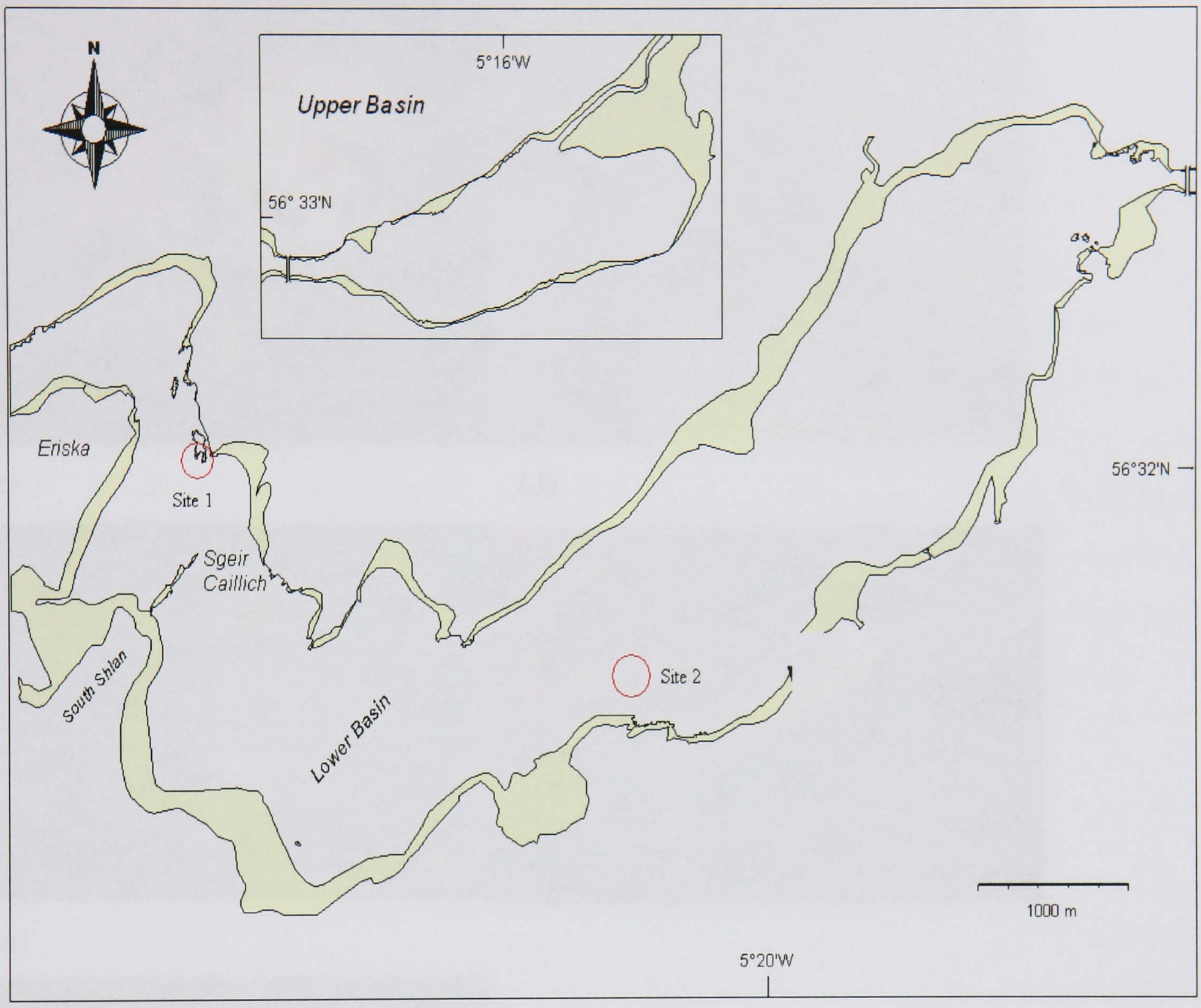


Figure 2.1 Sampling sites at Loch Creran, Argyll.

(a)

(b)

(c)

(d)

(e)

Figure 2.2 Sponges collected from Loch Creran (a); *Halichondria panicea* (Site 2),(b); *Suberites carnosus* (Site 2), (c); *Pachymatisma johnstonia* (Site 1),(d); *Suberites domuncula* (Site 2), (e) *Clione celata* (Site 1).

Seawater agar (SWA; 1 litre seawater and 15 g Bacteriological Agar No. 1 (Oxoid, Basingstoke, pH 6.8-7.0), Modified Emerson`s YpSS agar plates (YPSS; 4 g Yeast Extract, 10 g Soluble Starch, 16 g Bacteriological Agar No. 1, 750 ml artificial seawater, 250 ml Distilled water), Yeast Malt Extract Agar (YME; 4 g Yeast Extract, 10 g soluble starch, 4 g dextrose, 20 g NaCl, 20 g Difco Bacto agar), Tryptone Seawater Agar (TSA; 1 litre seawater and 5 g tryptone and 15 g Bacteriological Agar No. 1, pH 6.8-7.0) and Nutrient-Glycerol-Ferric Agar (NGF; 13 g Nutrient agar, 1% glycerol, 1mM $Fe_2(SO_4)_3$). All microbial media were incubated at 27°C for 5 days to up to 4 weeks. 100 randomly colonies were purified by streaking onto fresh media. Single colonies grown on these plates were transferred to fresh agar plates and further purified by streaking and re-streaking. Isolation was carried out immediately the sponges reached the laboratory. These were repeated whenever new sample of sponges reached the laboratory. Purified isolates were also stored on agar slant at 4°C. Each isolate label was designated according to the medium used for isolation such as MA, NGF or YPSS, respectively, followed by the isolate number.

2.1.3 Identification and characterization of the bacterial isolates

2.1.3.1 Gram-staining and micromorphology

Gram stains were performed as described previously (Smibert and Krieg, 1994). All of the isolated strains were examined using Gram-staining procedures. For this, a bacterial colony was diluted with sterile distilled water (SDW), spread onto a microscope slide and allowed to air dry in the lamina air flow hood, then heat fixed. The smear was then stained with flooded crystal violet (2.3% (w/v)) for 1 min and then rinsed with tap water. The slide was flooded with iodine solution for 1 min, again washed in tap water and a few drops of de-colourizer (acetone) were added onto the smear for 5 – 10 s or until de-staining was completed. The slides were rinsed thoroughly to wash off the decolourizer and then flooded with basic fushin stain for 1 min. The slides were finally rinsed with tap water and left to air dry. Slides were observed under a light microscope (Olympus BH2) at x1000 magnification. In addition, isolates were tested for Gram reactions using non-staining (Potassium hydroxide (KOH)) method (Buck, 1982). A drop of 3% (~ 10 µl) aqueous KOH was place on a slide. A visible amount of bacteria from an agar culture was

transferred using a sterile loop to the drop of KOH. The cells and KOH was mixed thoroughly on the slide, constantly stirring over an area about 1.5 cm in diameter. The bacterial isolate were considered as Gram-negative when the bacterium-KOH suspension became significantly viscous emulsion or gels within 5-60 s. If no gelling is observed, the isolate is Gram-positive.

2.1.3.2 Phenotypic tests

The morphological, cultural and biochemical features of strain SC-AF were determined. Gram stain, fermentation, catalase activity tests were conducted (Singleton, 1999) and oxidase activity by Kovac`s reagent. Starch, casein, and gelatin hydrolysis, anaerobic growth, and Voges-Proskauer reactions were carried out (MacFaddin, 1979). Morphology, capsules and motility were observed microscopically.

2.1.3.3 Extraction of DNA

Genomic DNA was extracted using the prescribed protocol of the DNeasy tissue kit (Qiagen, Crawley, UK). The protocols for DNA isolation from Gram-positive and Gram-negative bacterial cultures were different according to the prescribed protocol. Total DNA was isolated from 10 ml of culture isolates after overnight incubation at 28 °C. Bacteria were collected from the supernatant by centrifugation at 8000 rpm for 5 minutes, washed with distilled water and centrifuged at the same speed and time. The quality of the DNA was electrophoresed in a Gibco, BRL (USA) horizontal gel system as described below. Two hundred Gram-positive isolates were then continued for total DNA extraction and sequencing. The total DNA extracted from the isolates was than analysed by 16S rDNA sequence after which PCR amplification with 16S rDNA primer pair 9F and 1492R.

2.1.3.3.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis, for visualizing and estimating the molecular size based on the relative mobilities for DNA samples, followed the basic protocol (Sambrook *et al.*, 1989). The DNA was examined by 1% (w/v) agarose gel electrophoresis in TBE (Tris-Boric Acid-

EDTA) buffer (44.5 mM Tris, 44.5 mM Boric Acid, 1 mM EDTA at pH 8.0) and stained with 0.1 μg/ml ethidium bromide (Sigma). A 1% (w/v) agarose/1× TBE buffer mixed was boiled in a microwave, and 0.5μg/ml ethidium bromide was added to the solution when it cooled down. The mixture was poured into a gel-making tray with a special comb, which was used to provide wells for loading of DNA samples. Ten microlitres of the DNA samples were mixed with 2 μl of 6X gel loading dye (1/6 v/v) [(60% (v/v) glycerol, 60 mM EDTA, 0.09% (w/v) bromophenol blue, 0.09% (w/v) xylene cyanol)] before being loaded into the gel. The electrophoresis was carried out in an electrophoresis tank, which was filled with 1× TBE until immersion of the gel. A molecular weight marker, 1 kb DNA ladder (Fermentas) was used to check the size of DNA fragment. Two μl of the marker was loaded onto the gel as routine. The electrophoresis was conducted at 100V for 30 min. The gel was checked on a UV transilluminator, and a photograph was taken with a UVP gel documentation system.

2.1.3.4 PCR for 16S rDNA sequencing

Purified DNA from the bacterial isolates was amplified with universal primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and eubacterium-specific primer 1492R (5'-TACGGTTACCTTGTTACGACTT-3') in a PCR reaction containing 5 μl of 10x *Taq* Buffer (Fermentas), 8μl of dNTP mix (1.25mM, Fermentas), 10 pmol of each primer (MWG-Biotech,Germany), 20 ng of genomic template DNA, and 1 U of *Taq* DNA polymerase (Fermentas) in a final volume of 50 μl. The amplification program was started with one initial denaturation step of 95°C for 4 min; 30 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and elongation at 72°C for 60 s. The final elongation step was extended for 7 min.

The amplification reaction was performed by using a BioRad icycler thermal cycler (BioRad, Hercules, California). 16S rDNA gene was partially amplified by PCR and approximately 1400 bp segment of the 16S rDNA gene was purified following the protocol of QIAquick Gel Extraction kit (Qiagen). The purified product was then concentrated using ethanol precipitation following the protocol (Sambrook *et al.*, 1989). One hundred μl

DNA was precipitated by adding 4 µl of 3M sodium acetate and 100 µl of 100% cold ethanol. The mixture was then, centrifuged at 13 000 rpm (Eppendorf, Helena Bioscience) for 30 min and the pellet washed in 70% cold ethanol. After wash, centrifugation continued for another 10 min at the same speed. All the ethanol was removed carefully by pipette. The remaining pellet was air-dried, re-suspended in 50 µl of Millipore Water or Tris-EDTA (diaminoethanetetra-acetic acid) (TE) buffer (pH 8.0).

2.1.3.5 Sequencing of 16S rDNA gene fragments

The 16S rDNA gene sequence was analysed for the identification of new organism producing isolates. The sequencing was performed by MWG-Biotech with universal primers of 9F and 1492R. The sequences obtained were aligned with those in the GenBank by using Basic Local Alignment Search Tool (BLAST) (Altshul *et al.*, 1990) to determine approximate phylogenetic affiliations.

2.1.4 Characterization of uncultured bacteria

2.1.4.1 Preparation of DNA from whole sponges

0.25 mg of sponge tissue was ground in liquid nitrogen until it became powdery. Then, the sponge sample was transferred into a 1.5 ml microcentrifuge tube. Genomic DNA was extracted using the prescribed protocol of the DNeasy tissue kit (Qiagen).

2.1.4.2 PCR for Denaturing Gradient Gel Electrophoresis (DGGE) analysis

The PCR product was prepared as above (2.1.3.4). The PCR products generated were amplified a second time with primers 341F-GC (5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG G CAC GGG GGG ATT ACC GCG GCT GCT GG -3'), derived from position 341 to 357 of the *E. coli* 16S rDNA gene (amplification of variable V3 region

of 16S rDNA) and 518R spanning *E. coli* positions 518 to 534 (5'- ATT ACC GCG GCT GCT GG – 3') in order to add a GC-clamp to the DNA amplicons for DGGE analysis (Muyzer *et al.*, 1993). The PCR protocol included one initial denaturation step for 3 min at 95°C, followed by 30 cycles at 94°C or 30s, 56°C for 30s and 72 ^{0}C for 1 min, and one final elongation step for 7 min at 72°C. Three independent PCR reactions were performed for each of the samples. All primers used in this study were provided by MWG Biotech. DNA quality was examined after each amplification by 1 % (w/v) agarose gel electrophoresis in TBE buffer as 2.1.3.3.1.

2.1.4.3 DGGE analysis

GC-clamped PCR products were separated by DGGE using Dcode system (BioRad) and 16 cm x 16 cm x 1 mm gel. Polyacrylamide gels consisted of 8% acrylamide/bis (37.5:1) (Acrylogel 2.6, 40% solution, Electran, BDH) and TBE buffer with denaturing gradients ranging from 35-60%. Denaturing acrylamide of 100% was defined as 7 M urea (Analytical grade, Fisher Scientific) and 40% formamide (Biochemical, BDH). The gradient was made using a gradient maker and a peristaltic pump (Pharmacia) calibrated to 5 ml/min flow rate. Tubing was put at the exit of the gradient maker, going through the pump and finally attached to a yellow tip, which was fixed between two glass plates. The gradient maker was primed to prevent air locks by filling the hole with water. Magnetic bars were placed in the chambers, the one with the tube being placed over the stirrer (highly density).

Before making the gradient, a 1 ml plug (2 ml of 0% denaturant solution, 0.1g/ml Ammonium persulphate (APS, Sigma), 4 µl N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma)) was poured to avoid any leaking at the bottom of the plates After 30 minutes, 56 µl of 10% APS and 4.5 µl TEMED were added to 12 ml of each denaturant solution, previously poured into their corresponding chamber (the tap of the gradient maker being turned off), the most denaturant solution being in the chamber near the tube. The tap was then opened and the pump switched on. Once the denaturing gradient gel had been poured, it was immediately overlayered with 1 ml of water and allowed to polymerize at room temperature for 45 min. The tubing was rinsed with distilled water through the pump.

The stacking gel monomer solution (5 ml of 0% denaturant solution, 50 µl of 10% APS, 6 µl of TEMED) was poured over the denaturing gradient gel after removing the water. The well forming comb was inserted, and the gel allowed to polymerize for 30 min at room temperature. The gel was then mounted onto the electrophoresis apparatus as specified by the manufacturer.

During gel polymerization, the buffer tank was filled with 280 ml of 25x TBE buffer and the 1x volume was made up to seven litres with distilled water. The buffer tank was then preheated at 65°C prior to loading the PCR products. The plates were fixed on the rig and placed in the tank. The percentage of the gradient was optimized from 0% to 100% gradient of urea and formamide before the electrophoresis was performed. After the optimization was done, the PCR products were loaded onto 40% to 55% gradient of urea and formamide gel at 20 µl for pure culture samples. Electrophoresis was performed for 15 min at 20 V and subsequently for 12 h at 50 V in TBE buffer, at a constant temperature of 60°C. Gels were stained with 10 µg/ml ethidium bromide in TBE buffer. The gel was checked on a UV transilluminator, and photograph was taken with a UVP gel documentation system.

2.1.4.4 Band extraction from DGGE gel

DGGE bands representing unknown organisms were excised from the gel with a sterile scalpel and left at 4°C overnight in dH_2O. Very small cuts from the bands were used as templates for the subsequent PCR reaction with the primer pair used previously for DGGE. The quality of the PCR products was examined by agarose gel electrophoresis in TBE buffer as 2.1.3.3.1.

2.1.4.5 Construction of 16S rDNA clone libraries

PCR products were prepared as above (2.1.3.4) and cloned in *E. coli* cell, XL1-Blue with pGEM-T Easy Vector Systems (Promega, USA). Ligation reactions were set up as described by the manufacturer`s protocols. A mixture of 5 µl of 2x Rapid Ligation Buffer, 1

μl of pGEM-T Easy Vector (50ng), 1 μl of T4 DNA ligase (3 Weiss units μl^{-1}), 2 μl of PCR products and 1 μl of sterile Milli Q water were mixed. A total of 10 μl of ligation reactions were then incubated overnight at 4°C for the maximum number of transformants. For a positive control, 2 μl of control insert DNA containing uncut plasmid replaced PCR products. Two μl of each ligation reaction was added to a sterile microcentrifuge tube on ice, and then 50 μl and 100 μl (for positive control) of barely thawed competent cells were carefully transferred to the tube and placed on ice for 20 min. The cells were heat-shocked for 1 min in a water bath at exactly 42°C. Then, the tubes were immediately returned to ice for 2 min. After adding 1 ml of SOC medium (Sigma), the tubes were incubated for 1.5 h at 37°C with shaking (~150 rpm). The cells were pelleted by centrifugation at 1000 g for 10 min, resuspended in 200 ml SOC medium, and 100 μl volumes spread onto duplicate plates of LB medium containing ampicillin (Sigma), IPTG (Sigma), and X-Gal (Sigma). Colony growth occurred at 37 ^{0}C overnight. Selection of white colonies from LB plates was continued for identification of insert DNA.

2.1.4.5.1 LB media and culture conditions

Luria-Bertani (LB) medium (1% (w/v tryptone. 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl, pH 7.0) was used as bacterial medium. Culture plates were made by adding 1.5% (w/v) agar to the LB medium. LB medium was supplemented with ampicillin (100 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside) for transformation culture. All the medium and equipments were autoclaved. *E. coli* was grown at 37°C overnight.

2.1.4.5.2 DNA Minipreps

Plasmid DNA of clones was purified using the prescribed protocol of the QIAprep Spin Miniprep (Qiagen) manufacturer. A selected single colony (white colony as positive clone) was inoculated into 5ml LB broth with ampicillin (100 μg/ml) and incubated at 37°C overnight. Then, 1.5ml of incubated solution was transferred to a microcentrifuge tube and centrifugation was done at 13000 rpm for 20 minutes. The supernatant was discarded; the

pellets were used for purification steps and obtaining high-copy plasmid DNA. Purified plasmid DNA was then continued to the digestion step. This will ensure the insert of DNA.

2.1.4.5.3 Digestion of DNA Minipreps

Insert sizes of positive clones were analyzed by employing the restriction enzyme, *Eco*R1 (Fermentas). In general, 0.5-2 μg of plasmid DNA was added to 1 μl 10x restriction buffer, 0.5 μl of *Eco*R1, 0.1 μl of RNase, and dH_2O was added to complete the total volume up to 10 μl. The mixed solution was incubated at 37°C for 1-2 hours; the digested DNA was analyzed by agarose gel electrophoresis as 2.1.3.3.1. All clones containing inserts of the correct size of 1400 bp were then sequenced. Sequencing of cloned 16S rDNA genes were obtained by using primers M12forward and M13reverse. Sequencing processed as in 2.1.3.5.

2.1.4.6 Phylogenetic analysis

Comparative sequence analysis of 16S rDNA genes obtained from clones, isolated bacteria and DGGE bands (from culture-independent and culture-dependent approaches) was done using neighbour-joining method with TreeView (Page, 1996), based on alignments from CLUSTAL W (Thompson *et al.*, 1994). The partial (about 450 bp) and full-length (~1500 bp) 16S DNA sequence were added to a database comprising more than 10 000 complete and partial 16S rDNA sequences from BLAST search.

2.2 CULTIVATION OF BACTERIA ISOLATES

2.2.1 Bacterial media and culture conditions

Some pathogenic bacterial strains were used as target strains to show anti-bacterial activity. They included two strains of methicilin resistant *Staphylococcus aureus* (MRSA) 9551; J2407; one strain of vancomycin resistant *Enterococcus faecium* VRE 788; and two

pathogenic *E. coli* strains EMG9 and ADL1. They were provided by the School of Life Sciences, Heriot-Watt University. A volume of 25 litres of diagnostic sensitive test (DST, Oxoid) agar medium was poured into 9 cm petri dishes (Greiner). All freshly poured DST agar media were allowed to dry for 24 hours at 37°C before using to prepare lawns of the target strains.

2.2.2 Cultivation methods

2.2.2.1 The air-membrane surface (AMS) cultivation method

The AMS cultivation method followed by the previous study (Yan *et al.*, 2003). Six different types of media were used, Marine Broth (MB), Nutrient Broth (NB), SYZ Broth, NGF Broth, Medium A-1 (0.2% Casein hydrolysate, 3% Tryptone soya broth (TSB), 70% Natural sea water) and Medium A-2 (0.2% Casein hydrolysate, 3% TSB, 0.2% yeast extract, 70% Natural sea water). A nylon membrane (Millipore) was placed over a small shallow dish of a slightly smaller diameter than the membrane itself. The dish had been pre-filled with different kinds of autoclaved (121°C, 15 min) sterile liquid media, as above. Approximately 8 ml of media per dish was used for each of the media used so that the membranes were in contact with medium on one side and air on the other side (Figure 2.3). The focus was on the Gram-positive bacterial isolates. Thus, a total of ten bacterial strains were used (*Bacillus* sp., *Bacillus firmus, Micrococcus rosea, M. luteus, B. hwajinpoensis, B. decolorationi, B. cereus, Paenibacillus* sp., *B. lincheniformis, B. subtilis*) and one Gram-negative bacterial strain, *Pantoea* sp., SC-AF. All of the bacterial strains then grew in marine broth for four days at 28°C. The cultures were then swabbed onto the surface of each membrane type before incubating at 28°C. After five days, the antimicrobial activities were tested as described in 2.4.

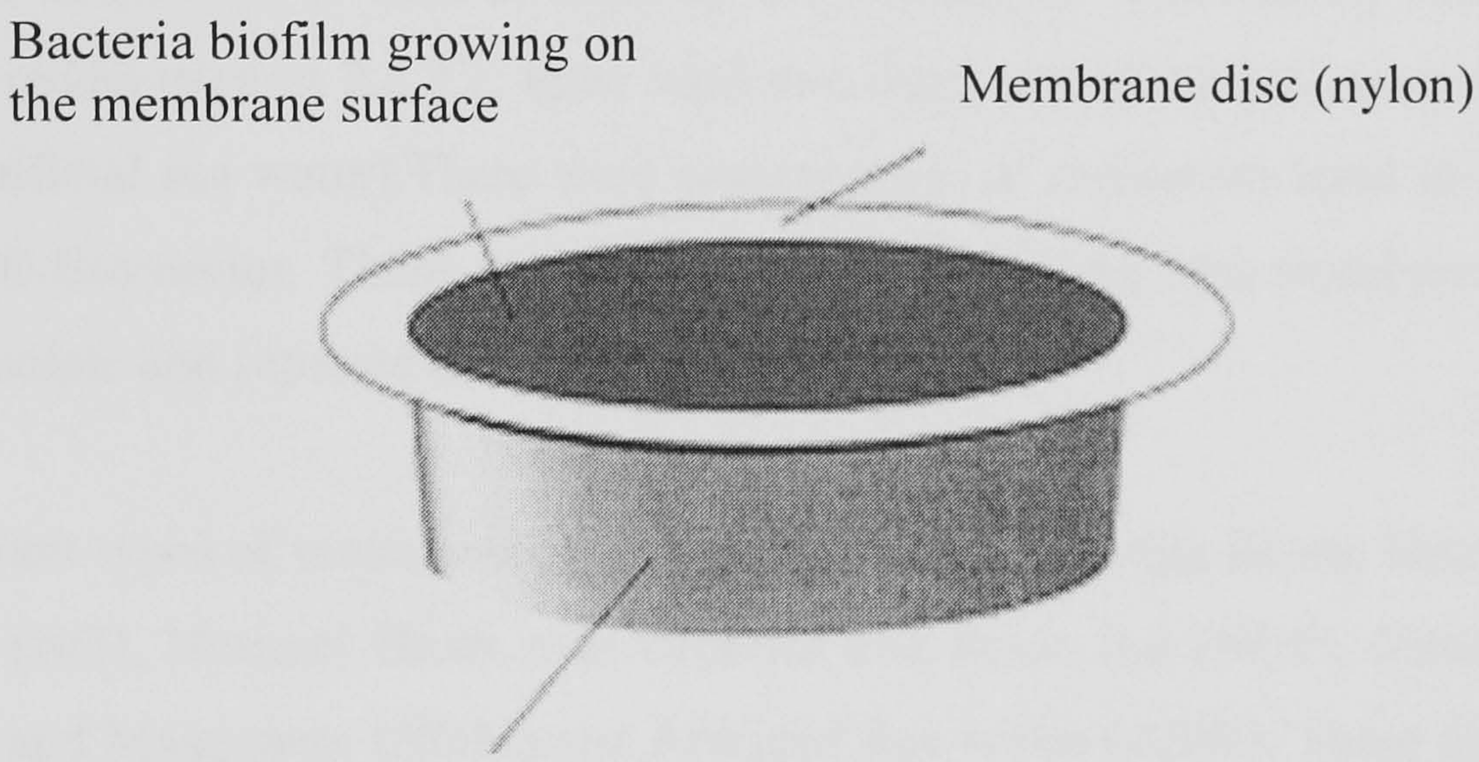


Fig. 2.3 The AMS bioreactor culture designed to allow bacteria to grow attached to the surface as a biofilm in contact with air.

2.2.2.2 ReacSyn®-Bioreactor

The ReacSyn®-Bioreactor provides a potential cultivation environment to mimic bacteria growing in the natural environment. The ReacSyn®-Bioreactor set up is to allow bacteria to grow attached to a surface as a biofilm in contact with air. The usage of a membrane filter, which is porous, will help the formation of a biofilm as well as the production of secondary metabolites. The laboratory has an established air-membrane surface (AMS) bioreactor with the production of antimicrobial compounds albeit in small-scale cultures. The ReacSyn®-Bioreactor is used to scale up the production of secondary metabolites. Apart from the media used in 2.2.2.1, there were two other media, natural sea water (NSW) and ASW (artificial sea water).There were several types of membrane used in the cartridge of ReacSyn®-Bioreactor. These were the way to ascertain the best membrane that could be holding isolate and separate them from the media.

Six different types of media were used, Marine Broth, Nutrient Broth, Nutrient Broth with Glycerol (NG), Nutrient Broth with Glycerol and Ferric ion (NGF), Nutrient Broth with Glycerol and Manganese (NGM) and Artificial Sea Water (ASW). Three different kinds of membrane were used: Polypropylene (1 and 25 μm (Millipore), Nylon (Whatman, 47mm), Cellulose Membranes (Millipore), and Cellulose nitrate (Millipore). All the membranes tested were put on the cartridges of the ReacSyn®-Bioreactor and autoclaved (121 ^{0}C, 15 min) before use (Fig. 2.4). Bacterial strains as in 2.2.2.1 were cultivated on the surface of semi-permeable membrane discs in contact with air and sterile medium (121 ^{0}C, 15 min), with a working volume of 40-45 ml. Strains were swabbed individually onto the surfaces of membranes and incubated at 28 ^{0}C for seven days. After incubation, the supernatant beneath the membrane was assayed and antimicrobial activities were tested as described in 2.4.

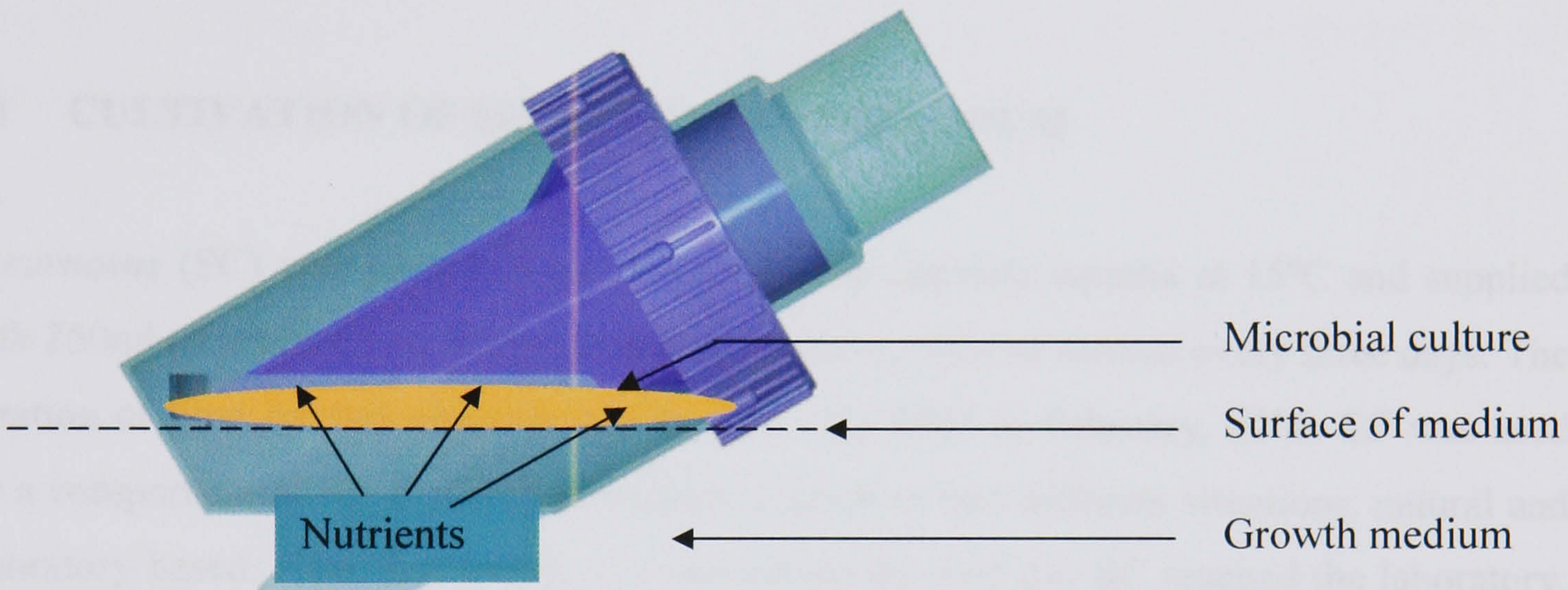


Fig. 2.4 ReacSyn®-bioreactor cultivation method designed to allow scale-up of media producing metabolites from the culture supernatant from underneath of the membrane (Biodiversity,UK).

2.2.2.3 Planktonic suspension Shake flask cultures

Six different types of media, as mentioned above, were used. All the bacterial test isolates as 2.2.2.1 were inoculated into sterile, 25 ml volumes of each of the different media in 100 ml Erlenmeyer flasks. The cultures were incubated on a rotary shaker at 200 rpm and 28°C for 7 days. After incubation, the antimicrobial activity was tested as described in 2.4. Nylon membrane was submerged into the liquid media of shake flask culture if needed.

2.3 CULTIVATION OF SPONGES IN AN AQUARIUM

S. carnosus (SC) was placed in an aerated 20-litre capacity aquaria at 15°C and supplied with 250ml of green algae, *Tetraselmis chui* or *Nannochloros atomus* every three days. The duration of time for this experiment was from July 2005 to February, 2006. SC was used for a comparison of the diversity of bacteria present in two different situations, natural and laboratory based. The first month was considered the first day SC reached the laboratory. Every two months, the sample of SC was taken for DGGE analysis until the 6^{th} month.

2.4 PRELIMINARY SCREENING FOR STRAINS PRODUCING ANTIMICROBIAL COMPOUNDS

All of the isolated strains which were Gram-positive were individually inoculated into 5 ml volumes of sterile marine broth 2216 (Difco, 121 $^{\circ}$C, 15 min) in Universal bottles for the preliminary screen. The bottles were incubated on a rotary shaker at 200 rpm and 28 ^{0}C for 5 days. A 1.5 ml aliquot from each culture was then aseptically transferred to a sterile microcentrifuge tube and centrifuged at 13,000 rpm for 10 minutes at room temperature. The antimicrobial activities were assessed from cell free supernatant (CFS) using the standard paper disc antibiotic assay or disc diffusion test (Mearns-Spragg, 1997). Then, sterile 6mm paper discs (Whatman) were saturated with CFS and left to air dry. The process was then repeated, with approximately 90 µl of supernatant, in total, being added to

each disc. The bacterial reference strains were swabbed on a DST (Diagnosis Sensitive Test) agar plate to form bacterial lawns. The saturated disc was then placed on the lawn to allow compounds in the disc to diffuse into the agar medium. The diameter of the inhibition zone was measured after plates were incubated at 28 ^{0}C or 37 ^{0}C for 12 hours. The CFS would be considered active only when the diameter was more than 6 mm.

The effect of different temperature on the activity of the CFS were determine by incubating 100 µl of the CFS at 40, 50 to up 100 ^{0}C for 5, 10, 15, 20, 30, 40 up to 60 min. Not only that, the CFS also autoclaved at 121 ^{0}C . The effect on the activity was determined by disc diffusion test as in 2.4.

2.5 IDENTIFICATION OF BIOACTIVE COMPOUNDS

2.5.1 Preparation of chemical extracts

CFS from AMS cultures was accumulated up to two litres. This took at least a week with 2000 caps of AMS cultures. Two litres of CFS was then evaporated completely in an evaporator (Buchi) at 37°C. The dried material was reconstituted in 1 ml volumes of organic solvents such as methanol (analytical grade, Fisher), butanol (analytical grade, Fisher), and ethyl acetate (analytical grade, Fisher). Different types of solvent were used in order to determine the polarity of the compounds. The compounds dissolved in the solvents were then tested by bacteria inhibition using the disc diffusion test as in 2.4. Additional discs, impregnated with 100 µl volumes of solvent were used as controls.

2.5.2 Preliminary assay of supernatant fractions

2.5.2.1 Ultrafiltration of supernatant

The supernatant of three strains *Bacillus subtilis*, SD-8, *B. licheniformis*, SC-43, and *Pantoea* sp., SC-AF were fractionated by molecular size using Amicon Ultra-15 (Millipore) with a cut-off 5 kDa. The filtrate was collected and filter-sterilized through a 0.2 µm filter

(Millipore). Any CFS remaining on the 5 kDa membranes was resuspended in the same volume of PBS buffer and centrifuged once again. Any CFS remaining on the membrane then resuspended again in the same volume of PBS buffer, filtered sterilized using a 0.2 μm filter.

2.5.2.2 Test of supernatant by ultrafiltration

The two supernatant filtrates (< 5 kDa and > 5 kDa) of each three strains were used and subjected to the disc diffusion test as in Section 2. 4. Preliminary results from the test gave input in term of the size of filtrates. This was then gave some information for the next step of purification method.

2.6 PURIFICATION OF BIOACTIVE COMPOUNDS

2.6.1 Solid phase extraction

The dried material of CFS as in 2.5.1, which was active according to the disc diffusion test, was chosen for further purification. Since methanol was the best solvent, then dried material of CFS was reconstituted in 10 ml of methanol. All the samples reconstituted in methanol were centrifuged at 13 000 rpm for 30 min to remove all the access materials. The supernatant was then evaporated completely in an air evaporator at 37°C. The dried material was reconstituted in 1 ml of distilled water and loaded into the column. Fractionation started by solid phase extraction (Sep-Pak Cartridge (Waters), Vac 6 cc silica; 1g) using gradients of H_2O and Acetonitril (Fisher) mixtures as the mobile phase (from 100% H_2O to 100% Acetonitril with 10% steps). Each fraction was tested for antimicrobial activity as before on the disc diffusion test. Additional discs were impregnated with 30 μl volumes of reconstituted sample before loading into the column used as controls.

2.6.2 High Performance Liquid Chromatography (HPLC) analysis of the extracted compounds

HPLC is a type of solid phase chromatography that uses high pressure to separate complex mixtures of compounds. A C18 solid matrix, which is the solid phase, was sealed in a high pressure resistant column. Solvent mixture or buffer, usually containing H_2O, is used as the mobile phase. The sample is injected along with the liquid mobile phase (which has been degassed) and passed through the column. Compounds with different lengths of carbon chains and polarity will be separated under different strengths of solvent or buffer. Hydrophilic compounds are usually eluted off the C18 column faster than hydrophobic compounds. The mechanisms involve a variety of interactions such as dipole-dipole interactions, van der Waals forces and hydrogen bonding. As the gradient is changed (the composition of the various solvent mixture or buffers being passed through the column) substances will be eluted off the column at different times i.e. compounds will partition into the liquid phase from the solid phase at different times (retention time). Solutes pass to a detector which has previously been calibrated with known quantities of the compound. The compound being assayed determines what type of column, buffers and detectors are used, and the gradient. HPLC grade water is often used as a diluent (deionised water > 18 megaohms/cm resistance (Mili-Q system)).

Fractions 70% H_2O : 30% MeOH and 60% H_2O : 40% MeOH, which was preliminary fractionated by C18 Sep-Pak® cartridge, were active against the target *M. luteus* strain. The two fractions were subsequently pooled and evaporated completely in rotovapor (BÜCHI) at 37°C. The dried material was reconstituted in 1 ml of distilled water. The reconstituted sample was centrifuged at maximum speed,13 000 rpm for 5 min and filtered using HPLC filter (0.2 μm PVDF membrane) with 1 ml syringe with the vial, and further purified using HPLC (Thermo, Finnigan Surveyor HPLC system). The sample was run over 100 min on the HPLC system. One ml of sample was loaded onto the column (phenomenex, Gemini 5μm C18) and run using the following programme: 40% isocratic 0-10 min; 40% MeOH : 60% H_2O → 60% MeOH : 40% H_2O 10-90 min; 60% MeOH : 40% H_2O → 100% MeOH 90-100min, at a flow rate of 0.2 ml min^{-1} (2 min/1%). The eluent gradient is shown in Table

2.1, the increasing proportion of eluent B removed the active compounds from the column. Both MeOH and H_2O used were HPLC grade (Fisher Scientific, UK).

Table 2.1 Eluent gradient programs for the fractionation of antimicrobial compounds produced by SC-AF

Time (minutes)	% Eluent A (H_2O)	% Eluent B (MeOH)
0	60	40
10	60	40
90	40	60
100	0	100

2.7 DETERMINATION OF POLYSACCHARIDES PROFILE BY HPLC

The basis for this assay was the partial ionization of carbohydrates at high pH and their subsequent separation by anion exchange. Separation was achieved by high performance anion exchange (HPAE) and carbohydrates are detected by a pulsed amperometric detector (PAD) which detects the electrical current generated by their oxidation at the surface of a gold electrode. This HPLC method was conducted by Heriot-Watt University technical support staff. The instrumentation was as follows: a Dionex PAD with gold electrode, a Gilson 302 pump, a Gilson 305 pump, a Gilson 802 Manometric Module, a Gilson 811B Dynamic mixer, a Hewlett Packard 1050 autoinjector, a Dionex eluent degas module, a Hewlett Packard Chemstation data handling system (HP3365) and a Dionex HPLC System.

The Gilson HPLC system provided the gradient pumping, sample injection and column separation of components. The column was a Dionex Carbopac PA-100 Guard column, 4 x 500mm with a Dionex Carbopac PA-100 4 x 250. The calibration standard was prepared by adding 1 g of glucose, fructose, sucrose (all from BDH), and maltose (Fluka), to a 100ml volumetric flask and diluting to volume with HPLC grade water. To a fifth flask 10mg of maltotriose (Sigma) and 1ml each of the glucose, fructose and sucrose stock solutions, plus 5ml of the maltose stock solution, was added and made up the volume to 100ml volumetric

flask with HPLC grade water, divided into 5ml aliquots and stored at –20 °C. 180 µl of the internal standard was added to 900 µl of test or standard solutions.

The eluent were as follow;

A: HPLC grade water sparged with helium for 15 minutes

B: HPLC grade water sparged with helium for 15 minutes with 44ml of concentrated sodium hydroxide added to give 500mM solution.

The eluent gradient program is shown in Table 2.2 and the retention times of the sugars in Table 2.3.

Table 2.2 Eluent gradient program for polysacccharides

Time (min)	% Eluent A	% Eluent B
0	20	80
13	30	70
20	50	50
25	75	25
28	100	0
31	100	0
31.1	20	80

Table 2.3 Retention times for sugar standards

Sugar	Retention time (min)
Glucose	3.96
Fructose	4.53
Sucrose	7.33
Cellobiose	8.87
Maltose	12.06
Maltotriose	22.60

2.8 PRELIMINARY STUDY OF PROTON (^{1}H) NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR) AND MASS SPECTROMETRY (MS)

HPLC purified compounds were dissolved in dueterium water (D_2O). Preliminary ^{1}H NMR was carried out using 200 MHz Bruker NMR. The signal was accumulated for 32 min.

The purified antimicrobial compounds were also identified using Mass Spectrometry (Clarus 600, Perkin Elmer) at University of Aberdeen for measuring the molecular mass of *Pantoea* sp., SC-AF.

CHAPTER 3 RESULTS

CHAPTER 3 RESULTS

3.1 DIVERSITY OF MARINE BACTERIA FROM THE SPONGES

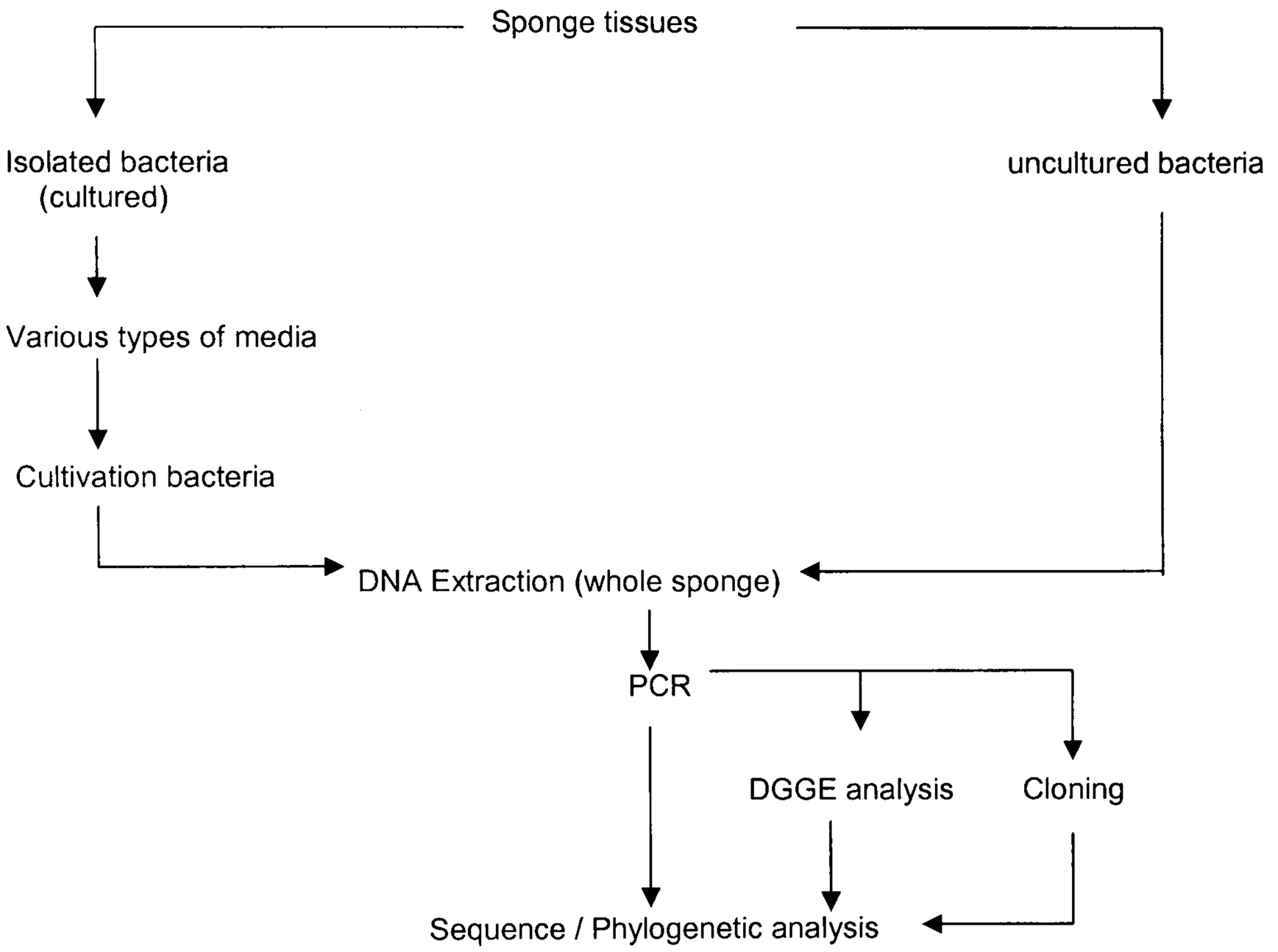


Figure 3.1 The approaches to analyse microbial diversity from the sponges.

DNA of the bacteria from the community associated with various sponges was extracted using both culture-dependent and culture-independent methods. Various types of media (as described in 2.1.2) were used to obtain Gram-positive bacteria (G +). DNA was taken through the polymerase chain reaction (PCR) to amplify the 16S rDNA gene fragment. The community structure was then evaluated through DGGE, cloning and sequencing.

3.1.1 Bacterial community characterized using cultivation-dependent method

There were 1020 isolates obtained using a range of bacteriological media, 235 isolates obtained from *Halichondria panicea*, 90 isolates from *H. bowerbanki*, 255 isolates from *Suberites domuncula*, 110 isolates from *S. carnosus*, 40 isolates from *Dysidea avara*, 12 isolates from *Chondrosia reniformis*, 39 isolates from *Axinella polypoides*, 83 isolates from *Clione celata*, and 156 isolates from *Pachymatisma johnstonia*. This study focused on Gram-positive bacteria, which accounted for approximately 20% of the isolates. Although results from the Gram-staining obtained supported by the KOH method (Buck, 1982) for unknown bacteria, confirmation results by using 16S rDNA gene sequencing were done. Appendix 1.0 shows the morphological data for Gram-positive bacterial isolates from the sponges after Gram-Staining. From nine species of sponges, 40.5 % of isolates formed beige colonies followed by 30.5% with white colonies, 6.1 % formed yellow colonies and only 22 % comprised other pigmented colonies of red, brown, tan, yellowish, grey, pink, red and greenish. Five % of isolates formed colourless colonies. Each species of sponge showed a different distribution of bacteria, with morphological studies showing that there were more rods than cocci.

In order to define the suitable media for the bacterial isolates, varieties of media were used such as Marine Agar (MA), Nutrient-Glycerol-Ferric (NGF) Agar, Nutrient Agar (NA), Seawater Agar (SWA), Modified Emerson`s YPSS Agar (YPSS), Yeast Malt Extract Agar (YME) and Tryptone Seawater Agar (TSA). Cultivation of isolates from four species of sponges namely *H. panicea*-2, *H. panicea*-3, *H. panicea*-5 and *H. panicea*-7 were used for selection of the media. Two different temperatures, 28°C and 37°C, were used to determine the best growth conditions for all the isolates tested. Among the type of media used for cultivation purposes, Marine agar (MA) was the best media for growth of all the isolates from the sponges at 28°C (Figure 3.2). Results indicated that the diameter of the colonies was significantly different between media.

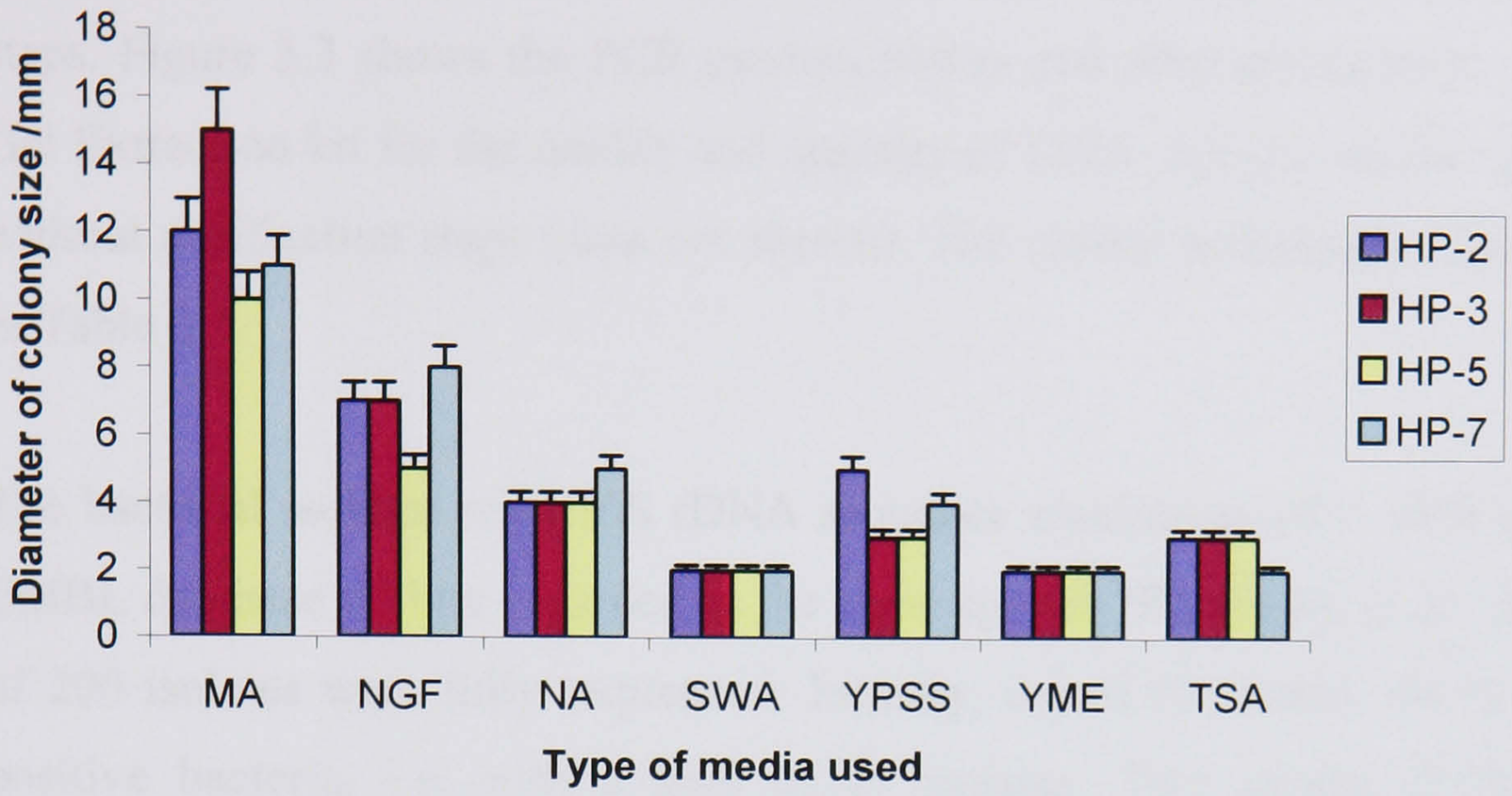


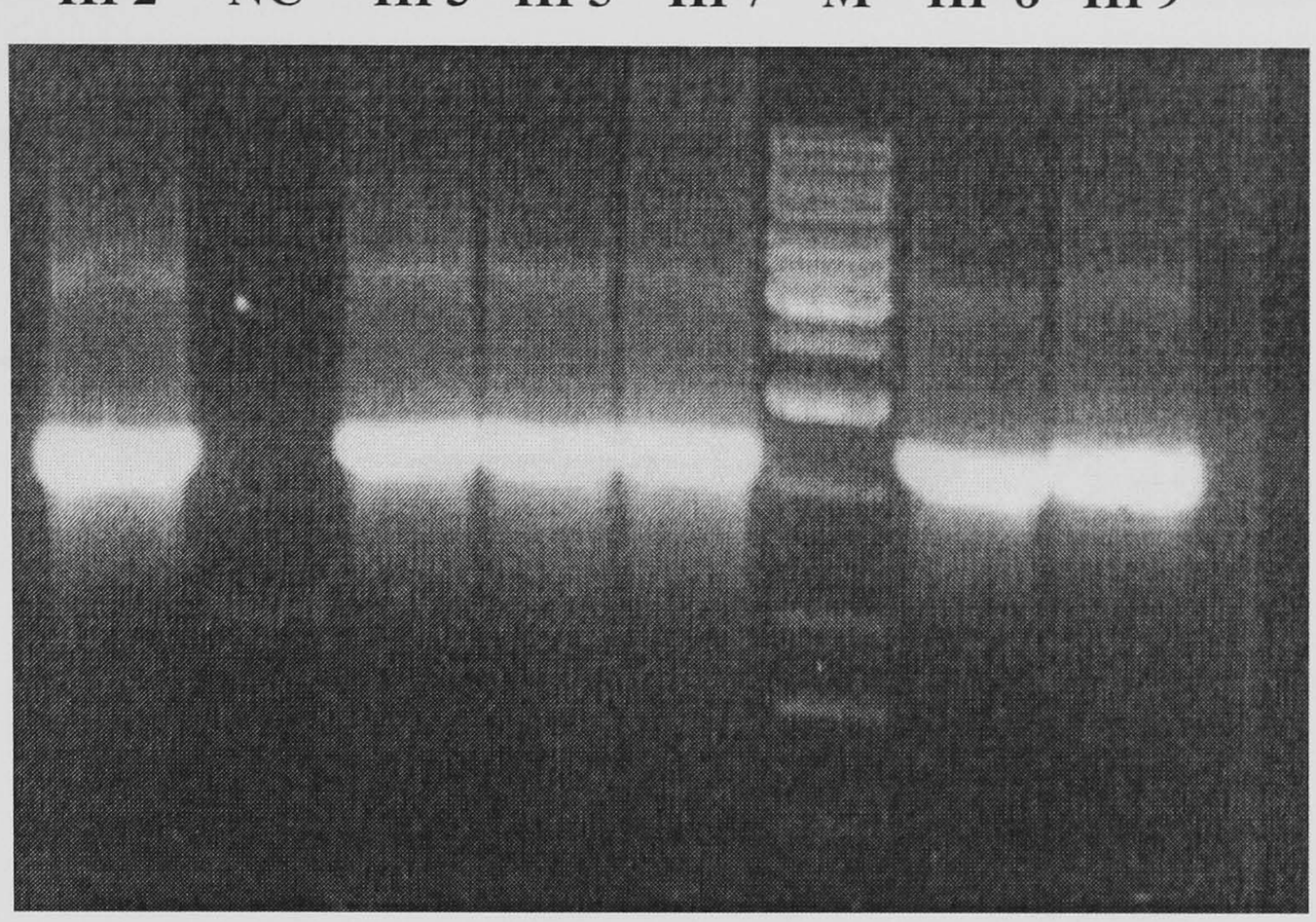
Figure 3.2 Selection of suitable media for cultivation of isolates. Diameters of the colony size were measured after seven days of cultivation of bacteria isolates. Four isolates of *H. panicea* (HP) were inoculated (HP 2,3,5,7) at a) 28°C and b) 37°C in seven different types of media, Marine agar (MA), Nutrient-Glycerol-Ferric agar (NGF), Nutrient agar (NA), Seawater agar (SWA), Modified Emerson's YPSS agar (YPSS), Yeast Malt Extract Agar (YME) and Tryptone Seawater Agar (TSA). Marine agar seemed to represent the best medium for isolation of sponge-associated bacteria at 28°C .

The total DNA extracted from 200 Gram-positive isolates was subsequently used as templates to amplify 16S rDNA primed by 9F and 1492R primer pairs. It was found that purification steps were crucial for obtaining expected sequence results in the following steps. Figure 3.3 shows the PCR product before and after purification using the Qiaquick Gel Extraction kit for the quality and quantity of DNA. All the results of sequencing failed without purification steps (data not shown). The closest homologous sequences are shown in Table 3.1.

The bacterial isolates with 16S rDNA sequence similarities of > 98% compared with the EMBL database will be regarded as the same species (Rehnstam *et al.*, 1993). Only 93 out of 200 isolates were fully sequenced. Initially, out of 93 strains, 44 % were from Gram-positive bacteria, i.e. with a total of 41 isolates. Two strains (2.1%) were from the Microccocaceae (*Micrococcus* sp. and *M. luteus*), two (2.1%) were from *Rhodococcus* sp. and *Kocuria* (*Kocuria* sp. and *K. rosea*) and the rest of the strains of the Gram-positive bacteria (39.8%) were from *Bacillus* and *Paenibacillus* (*Bacillus* sp., *B. cereus*, *B. macroides*, *B. baekryungensis*, *B. firmus*, *B. pumilus*, *B. licheniformis*, *Paenibacillus* sp. and *P. costantinii*). All isolates were from *H. panicea*, *S. domuncula*, *S. carnosus,* and *P. johntonia*; no Gram-positive strains were isolated from the other four species of sponges namely *Dysidea avara*, *Chondrosia reniformis*, *Axinella polypoides*, *Clione celata*. The remaining strains were all Gram-negative (Table 3.1).

After the 16S rDNA sequencing analysis, the bacterial strains were grouped (from Table 3.1). The diversity of strains from four species of sponges, *H. panicea*, *S. domuncula*, *S. carnosus* and *P. johntonia* are wide. Six strains (6/93 =6.45%) fell in the high G + C Gram-positive bacterial family group; 35 strains (35/93 =37.76%) were from the Low G + C Gram-positive bacterial family group; eight strains (8/93 = 8.6%) fell in the *Pseudomonadaceae* family group; four strains (4/93 = 4.3%) were from the *Enterobacteriaceae* family group; two strains (2/93 = 2.15%) were from the *Alteromonaceae* family group and the remaining strains were recovered in the alpha and gamma-proteobacteria groups. In particular, from the representative strains identified, almost 50% of the strains were from *Bacillus* which contributes to a group of Low G + C Gram-positive bacteria.

(a)



(b)

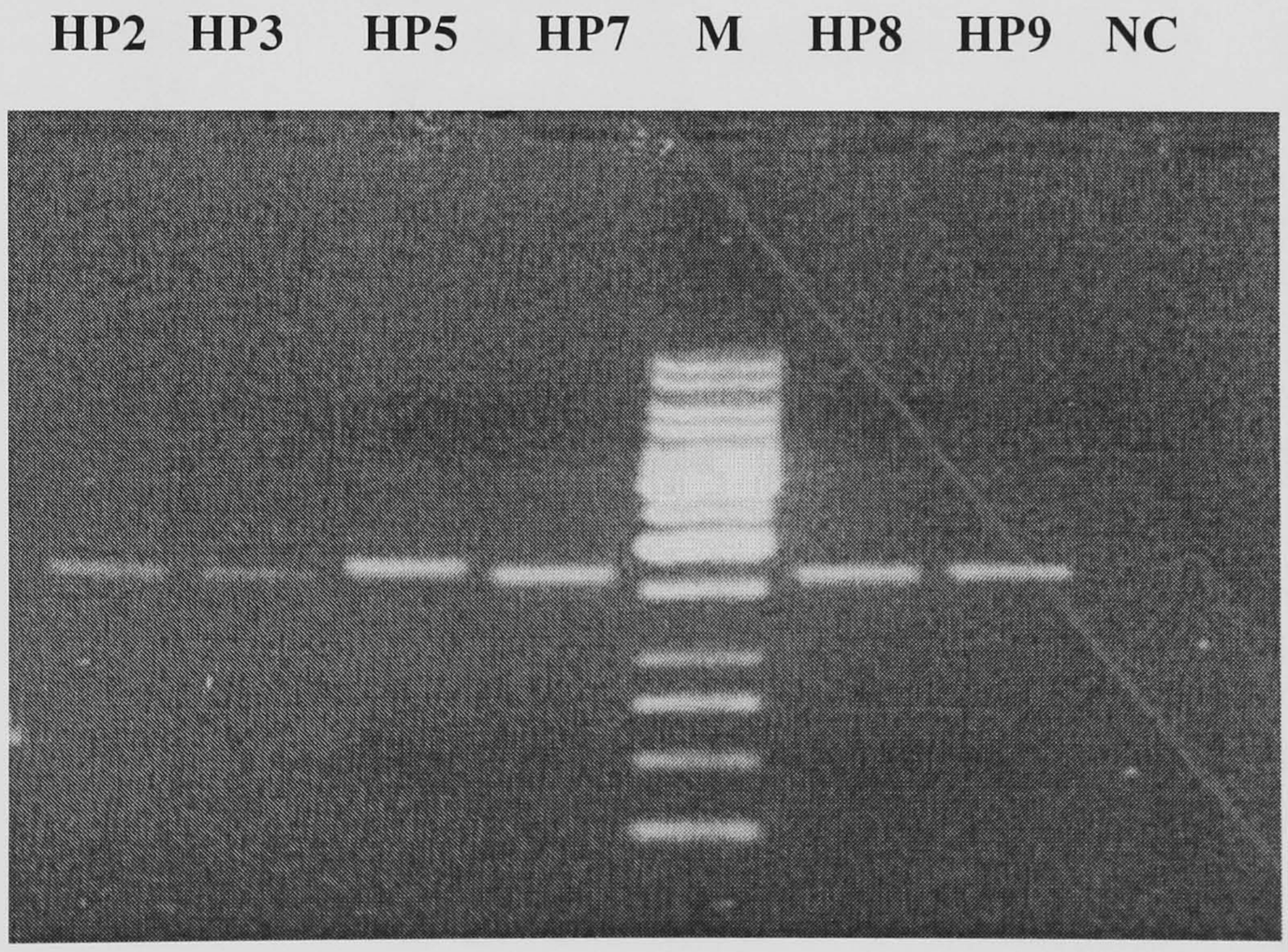


Figure 3.3 Comparison of PCR products before (a) and after (b) the purification method using the Qiaquick Gel Extraction kit. PCR products amplified from bacterial DNA using universal primers, 9F and 1492R on a 1% (w/v) agarose gel. M: 1 kb ladder (Fermentas) as a size marker. NC: negative control.

Table 3.1 Phylogenetic identification of strains isolated from four species of sponges using the 16S rDNA gene sequence analysis.

Strain ID[a]	No. of Isolates[b]	Length of partial sequence of 16S rDNA	Closest matching strain in EMBL	Accession number	% Sequence similarity
HP-2	1	550	Sponge bacterium isolates	AY948383	99
HP-3	1	614	*Rhodococcus* sp.	DQ371242	99
HP-5	1	613	*Vibrio splendidus*	AJ874367	100
HP-7	1	694	Marine arctic deep-sea bacteria	AJ557871	99
HP-8	1	538	*Aeromonas citrea*	X82137	99
HP-9	1	667	*Bacillus firmus*	AY833571	99
HP-5/6	2	548	*Micrococcus luteus*	AF542073	99
HP-13	1	712	*Microbacterium phyllosphaerae*	AJ277840	99
HP-15	1	554	Marine sediment bacterium	AY669172	99
HP-17	2	735	*Micrococcus* sp.	DQ409081	100
HP-22	5	809	*Bacillus cereus*	AY842872	99
HP-27	1	676	*Exiguobacterium* sp.	AY612767	100
HP-28	2	571	*Swanella* sp.	AY167276	100
HP-29	1	545	*Glaciecola mesophila*	AJ548479	99
HP-30	1	518	Uncultured *Vibrio* sp.	AY374409	100
HP-31	1	515	*Pseudoalteromonas porphyrae*	AY771715	99
HP-33	3	592	*Pseudoalteromonas* sp.	AY332141	99
HP-38	1	710	*Kocuria* sp.	AM179882	100
HP-43	1	590	*Bacillus pumilus strain JH7*	DQ232736	99
HP-51	3	600	*Vibrio* sp.	AB038024	99
HP-67	1	605	*Rhodococcus coprophilis*	RCU93340	97
HP-73	1	839	*Psychrobacter* sp.	DQ357011	99
HP-89	1	617	*Bacillus macroides*	AF501317	99
HP-99	1	862	Arctic sea bacterium	AF468380	100
HP-153	3	571	Uncultured bacteria clone	AY838470	100
HP-56	1	692	*Aeromonas* sp.	DQ200865	99
HP-100	1	760	Unidentified bacterium	AJ786785	97
HP-120	1	650	Marine bacterium	AF359548	99
SD-1	1	889	*Enterobacteriaceae*	AY538694	99
SD-2	1	571	*Psychrobacter glacincola*	AY165583	100
SD-5	1	564	Marine bacterium	AF359548	98
SD-7	2	590	*Paenibacillus* sp.	AY289507	99

Strain ID[a]	No.of Isolates[b]	Length of partial sequence of 16S rDNA	Closest matching strain in EMBL	Accession number	% Sequence similarity
SD-8	8	523	*Bacillus subtilis*	AY728013	99
SD-9	1	527	Bacterium JL	AY745842	99
SD-10	1	707	*Kocuria rosea*	DQ060382	99
SD-11	2	583	*α-proteobacterium*	AY562562	100
SD-12	1	583	Marine arctic deep sea bacteria	DJ557871	99
SD-18	1	645	Low G+C Gram-positive bacterium	AF34873	99
SD-19	1	594	Marine *γ-proteobacteria*	AF366050	99
SD-28	8	649	*Bacillus* sp.	DQ448800	100
SD-51	1	722	*Bacillus baekryungensis*	AY505500	100
SD-62	1	791	*Vibrio harveyi*	DQ146936	99
SD-69	2	912	*Pseudoalteromonas aliena*	AY387858	99
SD-193	2	546	*Bacillus thuringiensis*	AY795567	99
SC-1	1	620	*Swanella putrefaciens*	X81623	99
SC-3	1	653	Uncultured *Staphylococcus* sp.	AF467419	100
SC-6	1	661	*Pseudomonas* sp.	AY236959	99
SC-10	2	527	Mucus bacterium 97	AY654834	99
SC-11	1	538	Uncultured *Pseudoalteromonas* sp.	AF277504	99
SC-17	1	600	*Roseobacter gallaeciensis*	AJ867255	99
SC-47	2	910	*Pantoea ananatis*	AY530798	99
SC-50	1	705	*Bacillus anthracis*	DQ232746	99
SC-43	5	856	*Bacillus licheniformis*	AY842871	99
SC-AF	1	876	*Enterobacteriaceae* bacterium Smarlab	AY538694	99
PJ-11	1	930	*Paenibacillus toejonensis*	AF391124	99
PJ25	1	925	Bacterium NAS-14	AY729963	99
PJ-45	1	876	Unidentified bacterium	AJ786785	99

[a]HP-*Haliclondria panicea*, SC-*Suberitus carnosus*, SD-*Suberitus domuncula*, PJ-*Pachimastisma johntonii*
[b]Number of isolates grouped as the strain identified, the number of nucleotides for identification, the similarity of the sequence to the identified database species and the accession number of that species in the database are listed.

The Low G + C Gram-positive group comprised the dominant cultured bacteria for the strains isolated from sponges. The sequence data were then used in further phylogenetic analyses. Figure 3.4 and Figure 3.5 showed the neighbour-joining tree of the strains from this study compared to the reference strains, previously found in the marine environment obtained from the EMBL database.

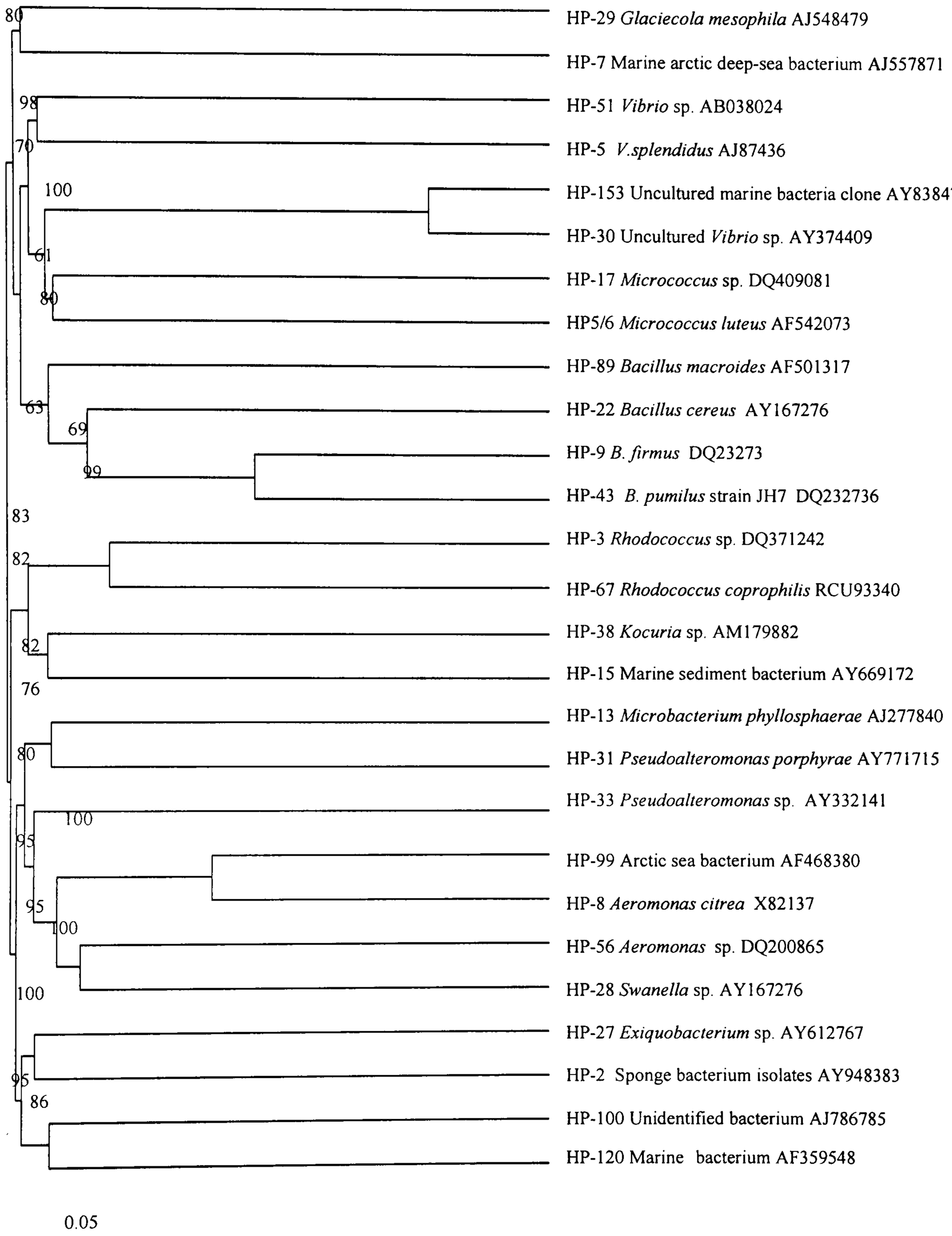


Figure 3.4 Neighbour-joining tree showing the relationship of the strains isolated in this study from HP with reference strains from EMBL (accession numbers are shown next to the named strains). The length of the sequence used for the construction was between 1300 – 1600 base pairs (with the exception of some strains which are ~500 bp). Bootstrap values shown of >50% support in an analysis of 1000 replicate tree. The scale bar represents 0.05 nucleotide substitutions per site.

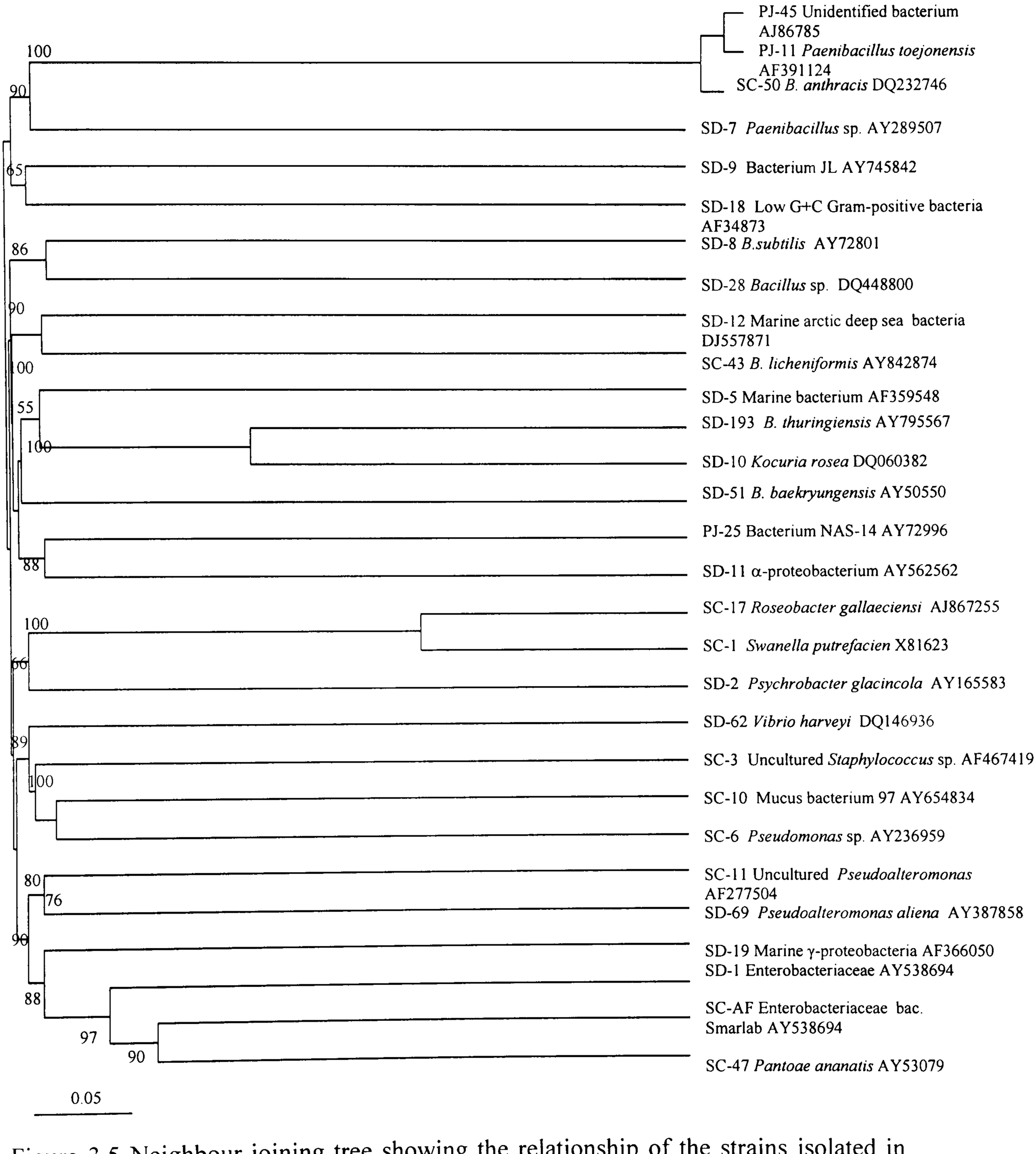


Figure 3.5 Neighbour-joining tree showing the relationship of the strains isolated in this study from SD, SC and PJ with reference strains from EMBL (accession numbers are shown next to the named strains). The length of the sequence used for the construction was between 1300 – 1600 base pairs (with the exception of some strains which are ~500 bp). Bootstrap values shown of >50% support in an analysis of 1000 replicate tree. The scale bar represents 0.05 nucleotide substitutions per site.

3.1.2 Bacterial community characterized using cultivation- independent methods

Two culture-independent methods, DGGE and Construction of 16S rDNA clone libraries, were used to study bacterial communities from the sponges. Total DNA extracted from the whole sponges of four species, *H. panicea* (HP), *Suberites domuncula* (SD), *S. carnosus* (SC) and *P. johntonia* (PJ), were primed by 9F and 1492R followed by PCR amplification. PCR products were run on an agarose gel to ensure that they were the correct size (approximately 1500 bp) and of enough quantity and quality for DGGE (Figure 3.6). A negative control (C) was also run to ensure that no contamination of the product was present. Several attempts were made to recover enough PCR products by optimising annealing temperature (40 to 60°C) for DGGE using a set of primer 341F-GC and 518R (data not shown). An annealing temperature of 56°C was optimal for both specificity and sensitivity. The variable regions (V3) of the 16S rDNA were separated on the polyacrylamide gel base on their sequences (one base different will give a different band) and as a result can be seen in Figure 3.7 to 3.9. Several of the bands run on the same distance and they were further resolved by running on a narrower ranging gradient (40-55% denaturant).

3.1.2.1 Intra-species diversity in microbial community composition

DGGE of PCR products, which were amplified from whole sponge DNA extracts, showed between seven and 20 detectable bands. There were between seven and 15 bands which could be detected in DGGE from 12 individuals of *H. panicea* (HP) samples (Figure 3.7). Although the banding patterns differed from one individual to another, some bands were shared among all these 12 HP samples (Table 3.2). Band 'a' was seen in all the samples except HP9-10, band 'b' occurred in all except for sample HP11 and HP12, band 'c' was present except for sample HP12, band 'd' was seen widely but not in sample HP4 or samples HP8 to 12; all samples had band 'e' and band 'f ' except for sample HP1. Band 'g' was recovered only from sample HP2, 8 to12 whereas band 'h' was from sample HP1- 2, and HP5-6. All eight strong bands ('a'-'h') were excised and sent for sequencing.

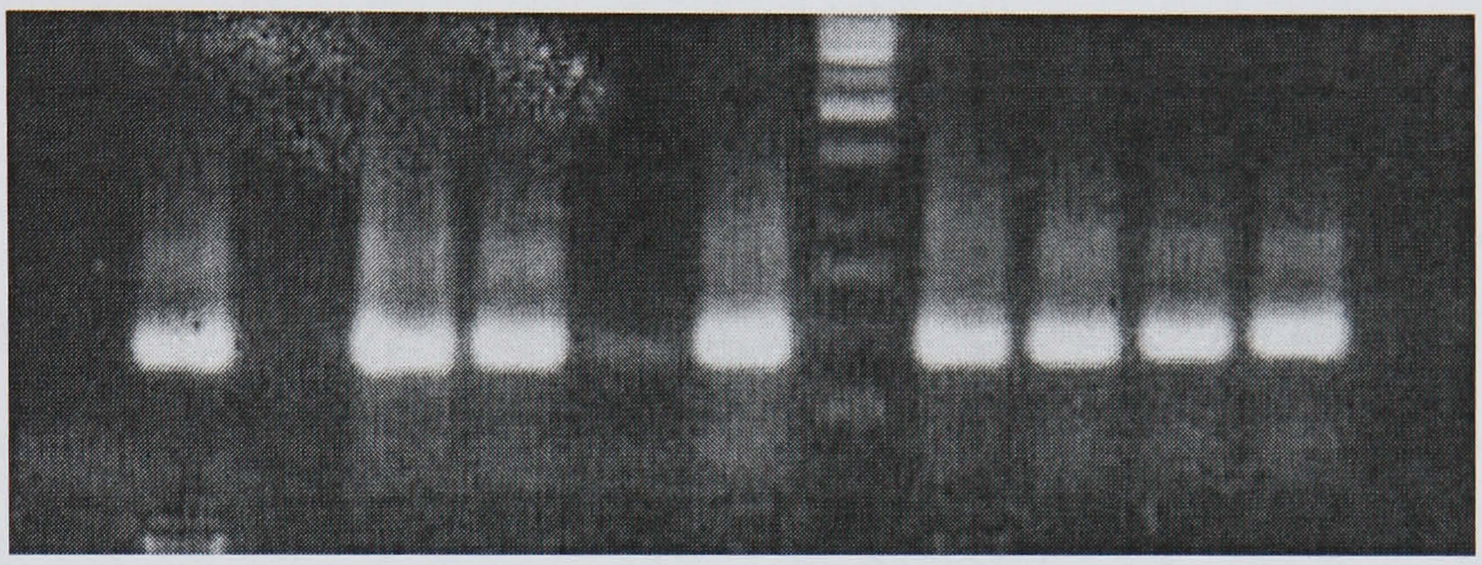


Fig. 3.6 PCR amplification products of the V3 region of the 16S rDNA gene of 11 individual sponge samples run on a 1% (w/v) agarose gel. C: negative control. M: 1 kb ladder (Fermentas, UK) as a size marker. HP: *Halichondria panicea*, SD: *Suberites domuncula*, SC: *S. carnosus*, PJ: *Pachymatisma johnstonia*.

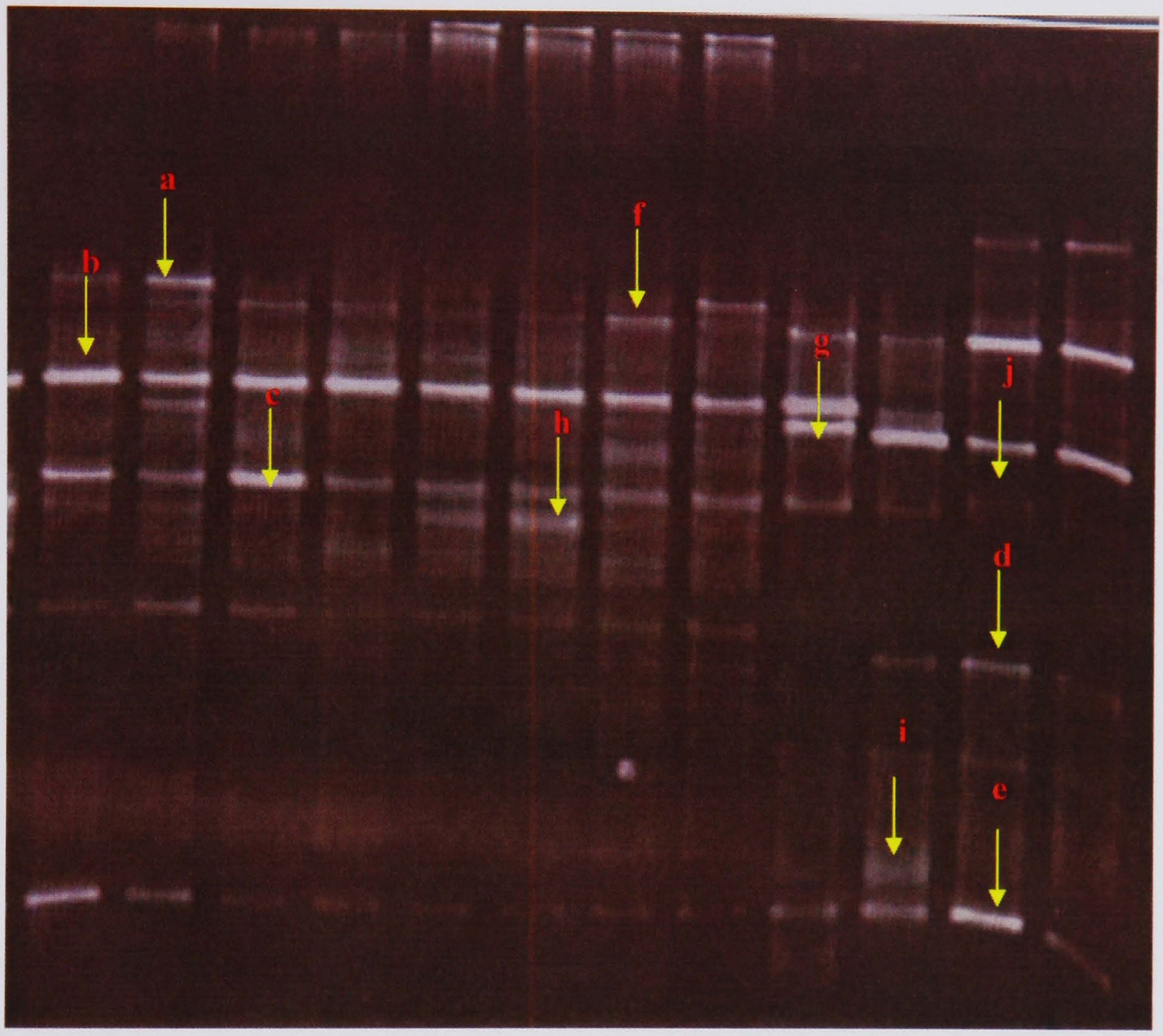


Figure 3.7 DGGE profiles of individual bacterial isolates from 12 individual sponges of *H. panicea* (HP). Each lane contains the amplified V3 region of the 16S rDNA products of various sponge associated bacteria from HP, run with a GC clamp on a polyacrylamide gel, with a linear gradient of 40-55% denaturants. The arrows show visible band of 'a' – 'j' (see Table 3.2 for band identifications). Each individual of HP seemed to have similar banding patterns except HP9-12. This suggested the existence of species-specific in bacteria associated with HP.

Table 3.2 Presence of DGGE banding patterns based on samples of *H. panicea* (HP)

Bands	Sample											
	HP1	HP2	HP3	HP4	HP5	HP6	HP7	HP8	HP9	HP10	HP11	HP12
a	√	√	√	√	√	√	√	√	√	-	-	√
b	√	√	√	√	√	√	√	√	√	√	-	-
c	√	√	√	√	√	√	√	√	√	√	√	-
d	√	√	√	-	√	√	√	-	√	√	√	-
e	√	√	√	√	√	√	√	√	√	√	√	√
f	-	√	√	√	√	√	√	√	√	√	√	√
g	-	√	-	-	-	-	-	√	√	√	√	√
h	√	√	-	-	√	√	-	-	-	-	-	-
i	-	-	-	-	-	-	-	-	-	-	√	-
j	-	-	-	-	-	-	-	-	-	-	√	-

All the strong bands were common to some of the samples such as band 'a', 'b', 'c' and 'g' and may simply reflecting number of isolates involved in the sponges community as well as the diversity of the bacteria in individuals of sponges. Two specific bands (band 'i' and 'j') were obtained from sample HP10 and HP11. Some of the bands were really faint and very close to each other. It seemed that each individual of HP had similar banding patterns except HP9-12. This suggested the existence of species-specificity in bacteria associated with HP. The same phenomenon was observed from Figure 3.8 of *S. domuncula* (SD) and *P. johntonia* (PJ) samples, and Figure 3.9 of *S. carnosus* (SC) samples.

DGGE samples prepared from *S. domuncula* (SD) were observed to have between four and nine detectable bands (Figure 3.8). The migrations of the five individual sponges, *S. domuncula* with SD1 to 5 were almost the same, and the samples consisted of four to eight bands, whereas four were very strong bands (band 'a'-'d') (Table 3.3). The similarity of the banding patterns indicated the existence of species-specificity from each of the individuals of *S. domuncula*. Almost all the strong band patterns obtained from *S. domuncula* were the same from one sample to the others. This indicated that there are some of the bacterial community represented by these bands that could be stable *S. domuncula* associated bacterial dominance in the sponges. Band 'e' was specific for sample *S. domuncula* from SD5. As for band 'f ', they were shared by sample SD2 and SD5 only. Lane 6-15 of ten individual samples of *P. johnstonia* (PJ1-10) produced four to nine detectable bands. Out of them, they shared similar bands of band 'b' and 'i ' from all the samples. Different phenomena were observed from each individual of *P. johnstonia*. Similar banding patterns were obtained only from PJ1, PJ4-5 and PJ10. The rest of the *P. johnstonia* samples had different banding patterns which indicated the existence of predominant bacteria associated with the *P. johnstonia* samples. Prominent DGGE bands were excised (band 'a'- 'i') and sequenced to gain insight into the identities of the predominant bacterial populations.

DGGE samples prepared from *S. carnosus* (SC) were observed to have 15 detectable bands from *S. carnosus* samples (Figure 3.9). All the samples had the same strong bands of bands 'a', 'b' and 'd', respectively (Table 3.4). The similarity of banding patterns from each of *S. carnosus* samples indicated the existence of species-specificity in bacteria associated with

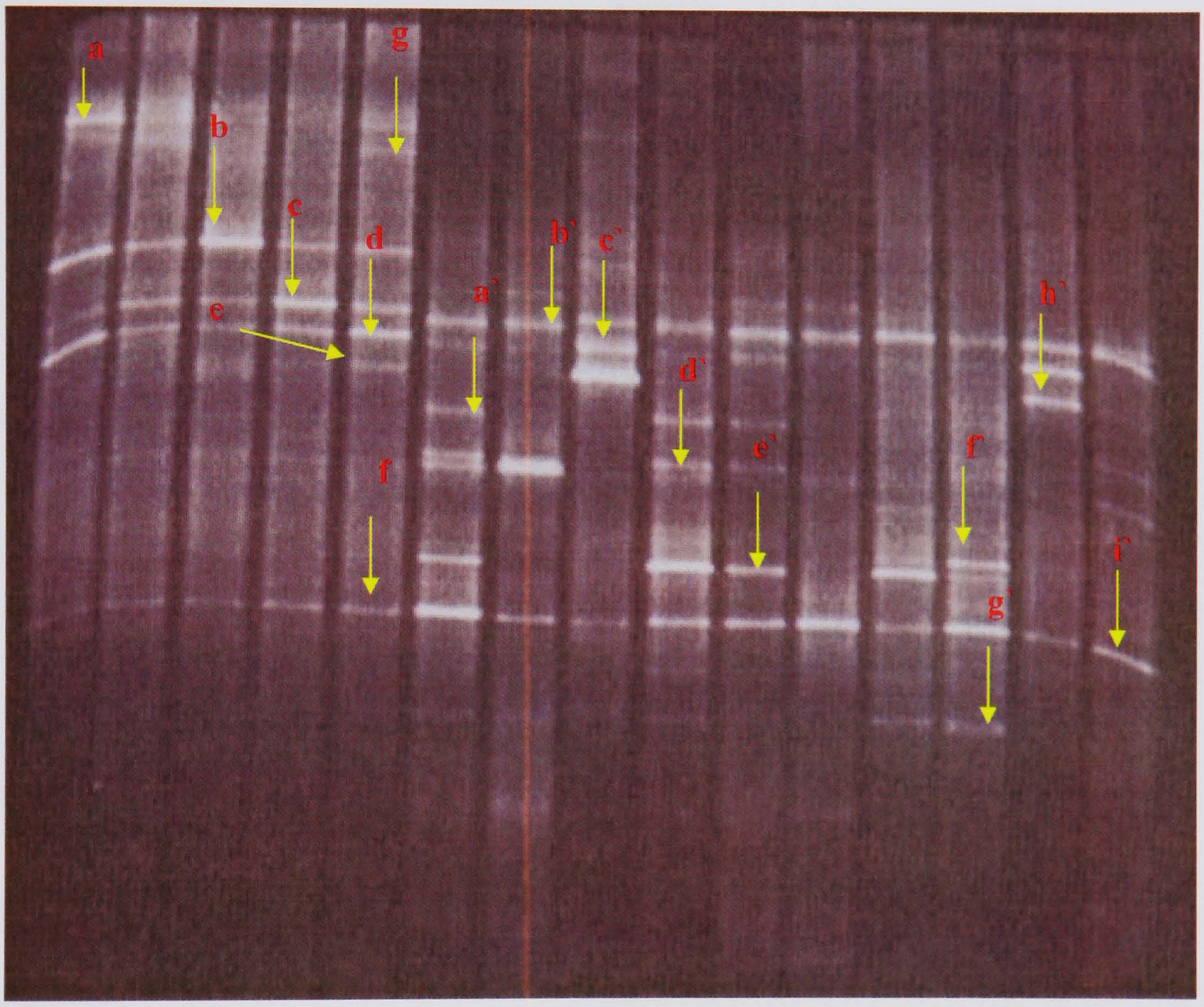


Figure 3.8 DGGE profiles of five individual bacterial isolates from the sponge *S. domuncula* (SD1-5), and ten individuals of *P. johntonia* (PJ1-10). Each lane contains the amplified V3 region of the 16S rDNA products of various sponge associated bacteria from SD and PJ, run with a GC clamp on a polyacrylamide gel, with a linear gradient of 40-55% denaturants. The arrows show visible bands 'a' – 'g' for SD samples and 'a``-'i`` for PJ samples (see Table 3.3 for bands identifications). SD1-5, PJ-1, PJ-4 & -5 and PJ-10 showed similar banding patterns indicating the existence of species-specificity among the individuals of SD and PJ.

Table 3.3 Presence of DGGE banding patterns from samples of *S. domuncula* (SD) and *P. johntonia* (PJ).

	Sample														
Band	SD1	SD2	SD3	SD4	SD5	PJ1	PJ2	PJ3	PJ4	PJ5	PJ6	PJ7	PJ8	PJ9	PJ10
a	√	√	-	-	√										
b	√	√	√	√	√										
c	√	√	√	√	√										
d	√	√	√	√	√										
e	-	-	-	-	√										
f	√	√	√	√	√										
g	-	√	-	-	√										
a`						√	-	-	√	√	-	-	-	-	-
b`						√	√	√	√	√	√	√	√	√	√
c`						√	√	√	-	√	-	-	-	√	√
d`						√	√	-	√	√	√	√	√	-	-
e`						√	-	-	√	√	-	√	√	-	-
f`						-	-	-	-	-	-	-	√	-	-
g`						√	√	-	-	-	-	-	√	√	-
h`						-	-	√	-	-	-	-	-	√	-
i`						√	√	√	√	√	√	√	√	√	√

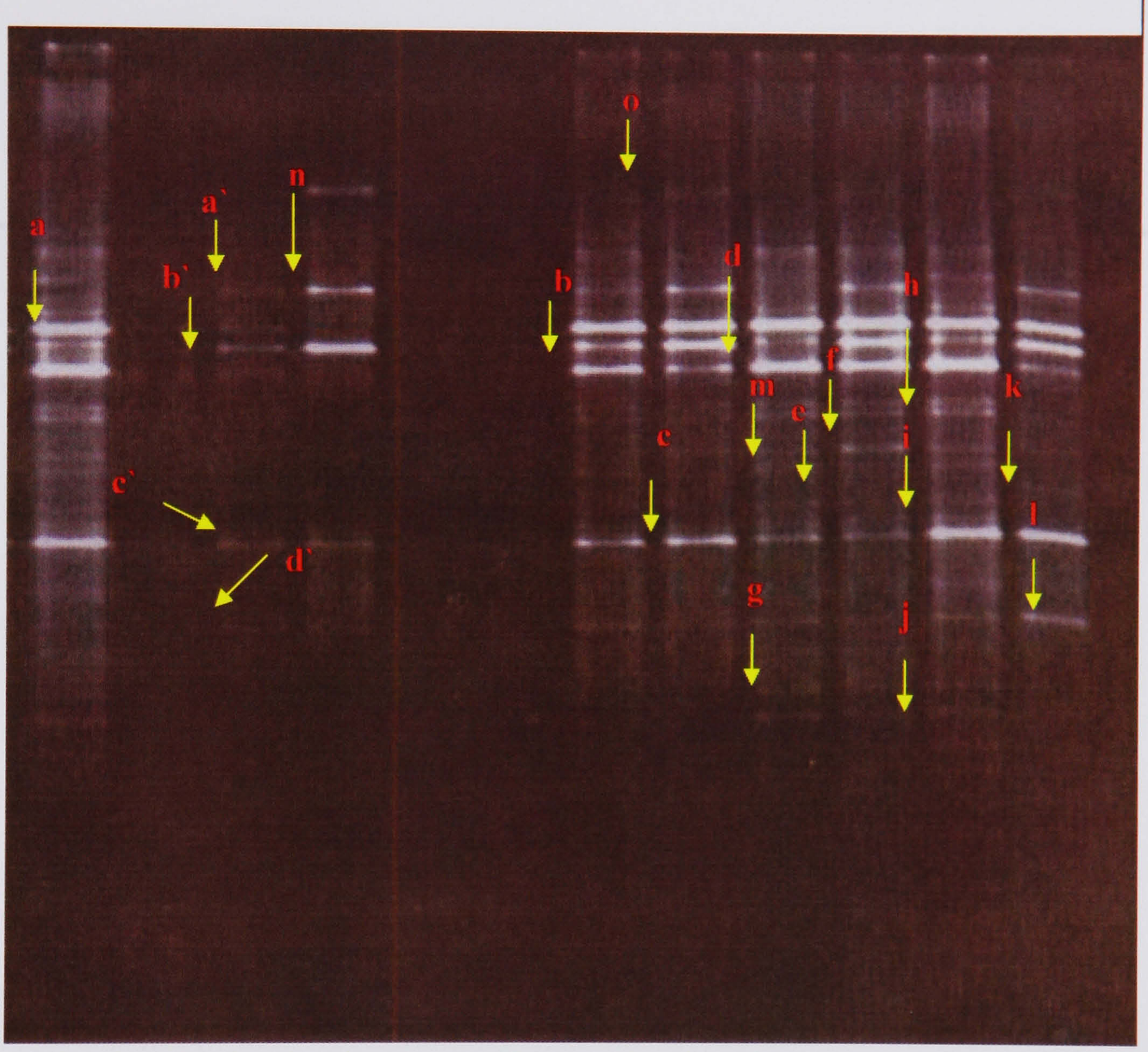


Figure 3.9 DGGE profiles of eight individual bacterial isolates from the sponge *S. carnosus* (SC1-8). Each lane contains the amplified V3 region of the 16S rDNA products of various sponge associated bacteria from SC, run with a GC clamp on a polyacrylamide gel, with a linear gradient of 40-55% denaturants. C1 and C2: control from seawater sample. The arrows show visible bands 'a' – 'o' from samples of SC and 'a``-'d`` from seawater sample (see Table 3.4 for band identifications). Each individual of SC seemed to have similar banding patterns. This indicated the existence of species-specificity in bacteria associated with SC.

Table 3.4 Presence of DGGE banding patterns based on samples of *S. carnosus* (SC)

	Sample							
Bands	SC1	SC2	SC3	SC4	SC5	SC6	SC7	SC8
a	√	-	√	√	√	√	√	√
b	√	√	√	√	√	√	√	√
c	√	√	√	√	√	√	√	√
d	√	√	√	√	√	√	√	√
e	-	-	-	-	√	√	-	-
f	√	√	√	√	√	√	√	√
g	√	√	√	√	√	√	√	√
h	√	√	√	√	√	√	√	-
i	√	-	-	-	√	√	√	-
j	√	-	-	-	√	√	√	-
k	-	-	-	-	-	-	-	√
l	√	√	√	√	√	√	√	√
m	√	-	√	-	√	√	√	-
n	√	√	-	√	-	√	-	√
o	-	√	-	√	-	-	-	-

S. carnosus. The same phenomena were observed from three other species of sponges mentioned above. Apart from that, the amplified V3 region showed different amount of DNA. A total of six strong bands ('a'-'d', 'n'-'o') were excised from all the samples of *S. carnosus* and used for sequence analysis. Band 'k' was the specific band from sample SC8. C1 and C2 were from seawater samples, as a control. It was clearly observed that only a few isolates were involved in the seawater. Four faint bands ('a'-'d') from the seawater samples were then sequenced to verify the presence of bacteria from the seawater environment not only from the sponges. The banding pattern from the surrounding seawater had fewer major bands, and many minor bands were present over a narrower gradient range than in the sponge samples. Common bands can also be seen between seawater samples and the sponge samples, suggesting that certain bacteria are present in both samples.

In the figures illustrating the DGGE analysis results, bands that were identified by 16S rDNA gene sequencing were indicated by arrows. The profile of bacterial communities showed significant differences between sponge *H. panicea, S. domuncula, S. carnosus and P. johnstonia*. Several attempts were made to recover enough DNA from the gels to allow PCR amplification. Different DNA bands recovered from DGGE were sequenced using a set of primers, 341F-GC and 518R. Unfortunately, no sequenced results were obtained from more than 50 samples. As a result, a new strategy was designed which aimed at improving identification of the bacterial species detected by DGGE. Thus, a new pair of primers, 968F-GC and 1401R which generated PCR products with a size of approximately 450-bp was used. This pair of primers were expected to overcome problems resulting from 314F-GC and 518R which can generate PCR products with only approximately 180-bp. Although the banding patterns revealed using 968F-GC and 1401R were the same as the primer pair 314F-GC and 518R, used previously, several attempts were made to obtain sequences and no results were obtained.

3.1.2.2 Inter-species diversity in microbial community composition

As intra-species diversity of bacteria associated with *S. domuncula* and *P. johnstonia* was relatively low, but with *S. carnosus* and *H. panicea* relatively high, comparisons of community composition between sponges were made. Analysis indicated that in term of

banding pattern, the microbial community composition of all the samples was significantly different. The highest diversity among all the samples was from *S. carnosus* and *H. panicea*, with 15 detectable bands compared to 8 in *S. domuncula*, and 9 in *P. johnstonia*. Banding patterns of species *S. domuncula* and *P. johnstonia* showed significant differences (Figure 3.8). This indicated the species-specificity of the microbes in the sponges.

3.1.3 Comparison of the bacterial community using 16S rDNA cloning in S. carnosus

A 16S rDNA gene library was constructed from the *S. carnosus* (SC) associated bacterial community using culture-independent methods. It was observed that *S. carnosus* had a better bacterial diversity compared with the other sponge species (see 3.11), therefore the bacterial community in *S. carnosus* was further studied. In addition, *S. carnosus* was also cultivated in an aquarium. There were 200 clones obtained in the first batch of library construction. Fifty clones from *S. carnosus* samples were then verified for the gene insert by DNA Miniprep (2.1.4.5.2) and DNA digestion using *Eco*R1 (Figure 3.10). Eleven sequence profiles were obtained and compared with BLASTN searches in Genbank. The best match to 11 phylogenetic types from the clones obtained was listed in Table 3.5. There was only one genus identified and documented which was *Synechococcus* sp. Four clones obtained; SC-2, SC-20, SC-17, SC-19 were from *Synechococcus* sp. followed by uncultured bacterial clone (four clones; SC-225, SC-211, SC12, SC13), unidentified sponge symbiont (one clone; SC-15) and unidentified bacteria DNA (two clones; SC-4, SC-13F). A phylogenetic tree was constructed using 16S rDNA gene sequences obtained after the BLASTN search (Figure 3.11), respectively. This group consists of *Synechococcus* sp, two of uncultured bacteria and two of unidentified bacteria which were not identified from culture-dependent methods. This analysis indicated that the microbial community composition of *S. carnosus* was significantly different from the culture-dependent method.

Table 3.5 Phylogenetic identification of 16S rDNA clone library from the sponge, *Suberitus carnosus* (SC).

No.	Strain ID	No. nucleotide	Closest matching strain in EMBL	Accession number	% Sequence Similarity
1	SC-2	1709	*Synechococcus* sp.	AY172800	100
2	SC-4	1289	Unidentified bacterium DNA	UBZ88569	99
3	SC-12	1366	Uncultured marine bacterial clone	DQ009318	99
4	SC-13	1761	Uncultured bacterial clone	DQ490036	98
5	SC-13F	1550	Unidentified bacteria DNA	UBZ88569	99
6	SC-15	1230	Uncultured sponge symbiont	AF434970	96
7	SC-17	1797	*Synechococcus* sp.	AY172800	100
8	SC-19	1852	*Synechococcus* sp.	AY172800	100
9	SC-20	1840	*Synechococcus* sp.	AY172800	100
10	SC-211	1494	Uncultured bacterium clone	DQ490036	99
11	SC-225	1287	Uncultured bacterium clone Sponge	AY948360	100

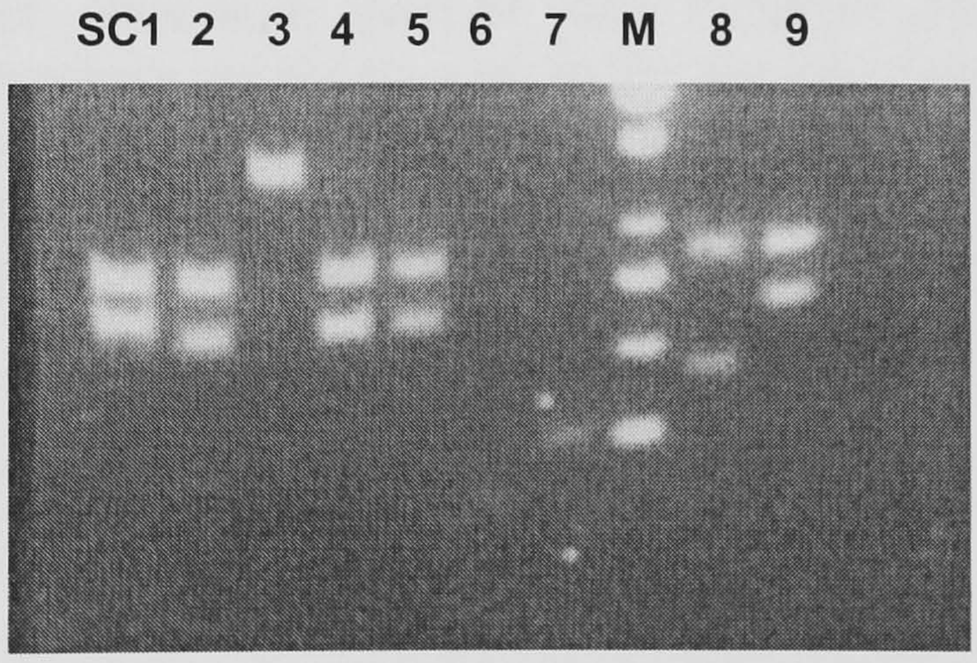


Figure 3.10 PCR products after purification of plasmids and digestion with *Eco*R1 restriction enzyme to verify the insert of the genes. *S. carnosus* (SC) 1, 2,4,5,9 show two DNA fragments which have restriction site for *Eco*R1. SC3 shows one DNA fragment which indicated only one restriction site for *Eco*R1. No insert for SC6 and SC7. M: 1 kb ladder marker.

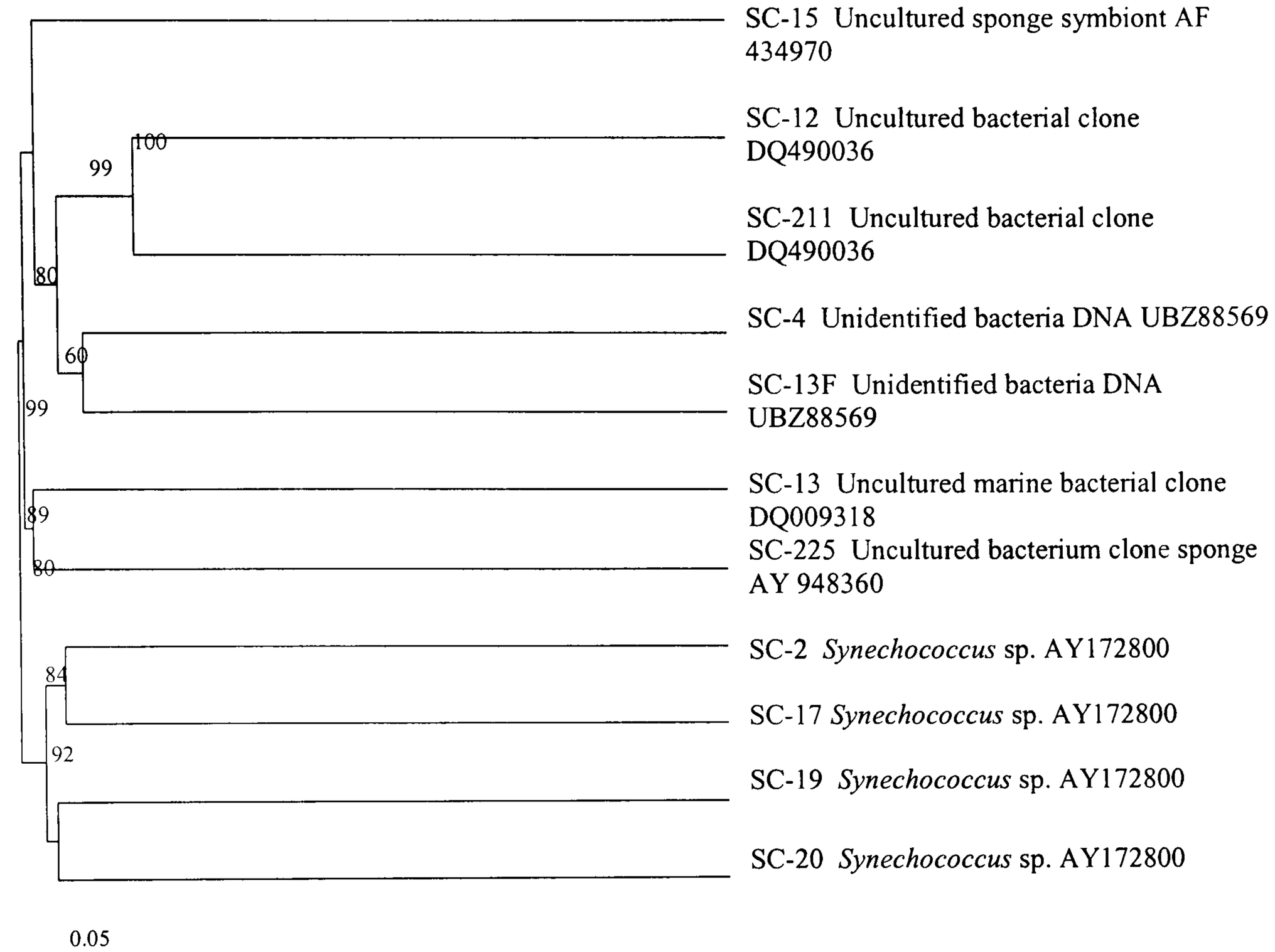


Figure 3.11 Neighbour-joining tree showing the relationship of the clones obtained in this study from SC with reference strains from EMBL (accession numbers are shown next to the named strains). The length of the sequence used for the construction was between 1300 – 1600 base pairs. Bootstrap values shown of >50% support in an analysis of 1000 replicate tree. The scale bar represents 0.05 nucleotide substitutions per site.

3.1.4 Characterization and comparison of bacterial communities associated with S. carnosus (SC) from the natural environment and an aquarium environment

In order to further understand the build-up of sponge associated bacterial communities, *S. carnosus* (SC) was artificially cultured in an aquarium, and the change of the bacterial community studied. *S. carnosus* was cultured for six months to allow a stable microbial community to establish. Seawater samples from the aquarium were used as controls (C: Figure 3.12). The total DNA of *S. carnosus* was extracted from the different samples after cleaning. As aforementioned, the amplicons produced by PCR were electrophoresed by DGGE (Figure 3.12). Initially, four dominant bands (a-d) indicating four different strains were obtained from all the samples regardless of the time. There were no significant differences (blue box) of the predominant bands when compared with the sample from the natural environment (Figure 3.9 in yellow box).

Apart from the presence of four dominant bands retrieved from the DGGE gel, there were significant difference between all the faint bands retrieved. Numerous bands disappeared after two months growth in the aquarium and all faint bands gradually disappeared after six months (band e-m). Only three faint bands were detected from aquarium samples whereas from the natural environment more than 14 bands were seen. Four of the dominant bands were then excised, and DNA extracted from the gel. The amplicons were sequenced. Several attempts were done to get the full sequence of the excised bands from DGGE gels. Unfortunately, sequence results were not successful. The DGGE patterns observed for *S. carnosus* from the natural environment were somewhat more complex than those obtained from the aquarium samples, and the latter contained a subset of the bands present in samples of *S. carnosus* from the natural environment.

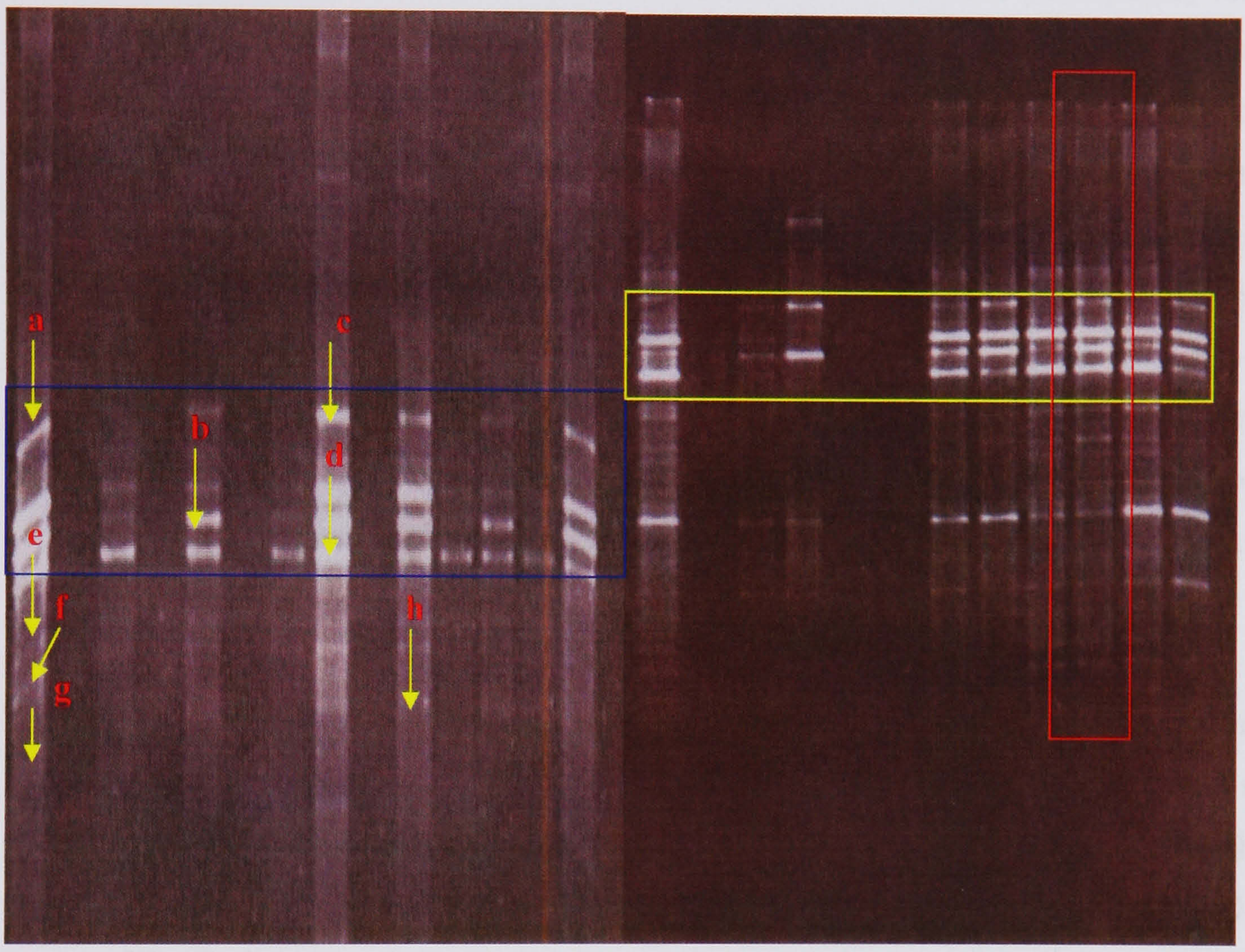


Figure 3.12 DGGE gel pattern from different parts of sponge, *S. carnosus* (SC) (a) after 2-6 months cultivation in the aquarium (b) from the natural environment. (a) SC9 was after two months, SC10 and SC11 after four months and SC12-14 after six months. C: control from the aquarium seawater. (b) SC6 (in the red box) of the same individual of SC on the first day of arrival in the lab (month 1). The fewer bands observed from an aquarium samples indicated that some of the bacteria associated with SC need the natural environment to grow.

3.2 PRODUCTION OF ANTIMICROBIAL COMPOUNDS BY SPONGE ASSOCIATED BACTERIAL ISOLATES

Most of the isolates, which were identified as Gram-positive, were subsequently screened for antimicrobial activities. In particular, after Gram-staining, 20% of the isolates (200 isolates out of 1020) were then used for the preliminary screening. The antimicrobial activities of the strains isolated from this study were then tested against each other. This indicated the existence of antagonism among the strains. Of all the strains tested, supernatants from only eight strains (*M. luteus*, HP 5/6, *B. firmus*, HP-9, *Bacillus* sp., SD-28, *B. cereus*, HP-22, *B. pumilus*, HP-43, *B. subtilis*, SD-8, *B. baekryungensis*, SD-51, *B. licheniformis*, SC-43, *Enterobacteriaceae* bacterium Smarlab, SC-AF) cultivated in marine broth showed antimicrobial activity against at least one of the strains isolated from the sponge. Strain names, bacterial strains used for the antagonism test and antimicrobial activities are listed in Table 3.6.

All of the strains showed inhibition activities against *Micrococcus* sp. The inhibitory activity of *B. licheniformis*, SC-43 and *B. subtilis*, SD-8 against various bacterial strains tested showed antagonism on Gram-positive bacteria. *B. licheniformis,* SC-43 showed consistent inhibition effects against diverse strains except against *B. subtilis*, SD-8, *K. rosea*, SD-10, *B. baekryungensis*, SD-51 and all the Gram-negative tested. *B. subtilis*, SD-8 displayed almost the same pattern of inhibition against indicator strains as *B. licheniformis*, SC-43. However, *B. cereus*, HP-22 and *B. pumilus*, HP-43 showed only inhibitory activity against *Bacillus* sp. and *Micrococcus* sp. Apart from that, HP-43 was active against *B. cereus*, HP-22.

Three Gram-negative strains, SC-43, SC-47, and SC-AF were studied for antagonism activity as well. All were only active against *Micrococcus* sp. These three bacterial strains were the only active strains, which could produce antimicrobial compounds. Interestingly, the morphology of these strains with a jelly-like appearance on the surface of the NGF agar medium drew my attention for further study even though they exhibited only slight inhibition toward *M. luteus*. It seemed that NGF agar medium was optimal for the production of jelly-like compounds within colonies. In contrast to NGF, nutrient agar and

Luria-Bertani (LB) agar, did not contribute to the production of the jelly-like colonies. This indicated that the mixture of glycerol and ferric ion are needed for the `jelly-like` consistency. These five strains (*B. cereus*, HP-22, *B. pumilus*, HP-43, *B. subtilis*, SD-8, *B. licheniformis*, SC-43, *Enterobacteriaceae* bacterium Smarlab, SC-AF) were then selected for further studies although HP-22, HP-43 and SC-AF showed very weak activities. The proportion of antimicrobial producing strains in this study was about 12%, which was the same as previously reported (Mearns-Spragg, 2000; Yan *et al.*, 2003). However, other studies demonstrated that 35% of strains from various species of seaweed and invertebrates from Scottish coastal waters produce antimicrobial compounds (Burgess *et al.*, 1999). NGF media was the best medium for some of the isolates to significantly enhance antimicrobial compound production as well as other secondary metabolites, such as red pigment.

Table 3.6 Antimicrobial activities of the strains isolated from sponges tested again each other.

Strains name	Sponge source	Indicator strains												
		HP-5/6	HP-9	SD-28	HP-22	HP-43	SD-8	SD-10	SD-7	SC-10	SD-51	SC-43	SC-47	SC-AF
M. luteus,HP-*5/6*	HP	-	-	-	-	-	-	-	-	-	-	-	-	-
B. firmus, HP-9	HP	1	-	-	-	-	-	-	-	1	-	-	-	-
Bacillus sp., SD-28	HP	1	-	-	-	-	-	-	-	1	-	-	-	-
B. cereus, HP-22	HP	1	-	2	-	-	-	-	-	1	-	-	-	-
B. pumilus, HP-43	HP	1	-	2	1	-	-	-	-	1	-	-	-	-
B. subtilis,SD-8	SD	5	-	4	3	-	-	-	2	4	4	-	-	-
K. rosea,SD-10	SD	-	-	-	-	-	-	-	-	-	-	-	-	-
Paenibacillus sp.SD-7	SD	-	-	-	-	-	-	-	-	-	-	-	-	-
B. baekryungensis, SD-51	SD	1	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus sp. SC-10	SC	-	-	-	-	-	-	-	-	-	-	-	-	-
B .licheniformis,SC-43	SC	8	-	4	2	2	-	-	2	6	4	-	-	-
Pantoea ananatis, SC-47	SC	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacteriaceae bacterium Smarlab SC-AF	SC	2	-	-	-	-	-	-	-	-	-	-	-	-

Antimicrobial activities were determined as inhibition zones around the lawn after overnight growth (mm, n=5). (-) = no activity. HP: *Halicondria panicea*, SD: *Suberites domuncula*, SC: *S. carnosus*

3.2.1. Production of antimicrobial compounds when grown in planktonic suspension culture

Five marine bacterial strains (*B. cereus*, HP-22, *B. pumilus*, HP-43, *B. subtilis*, SD-8, *B. licheniformis*, SC-43, *Enterobacteriaceae* bacterium Smarlab, SC-AF), which were active from 3.2, were grown using planktonic suspension culture for antimicrobial screening. None of the supernatants from shake flask cultures showed any antimicrobial activity against marine strains, as well as MRSA and VRE. This demonstrated that none of the strains selected produced antimicrobials when grown in the planktonic suspension culture. Further experiments showed that if all the five strains selected were grown in planktonic suspension culture for seven days, they formed a biofilm on the inner wall of the shake flasks. Apart from that, no colour change was observed.

If cell-free supernatant (CFS) of the same strains (*B. licheniformis*, SC-43 and *B. subtilis*, SD-8) from AMS culture were added to the shake flask cultures, the colour of the liquid cultures changed to red after cultivation for a further two days. Antimicrobial activity of the supernatant from both cultures was then tested. The cell-free supernatant from biofilm culture established on nylon membranes could elicit the production of antimicrobial compounds by corresponding planktonic suspension culture (Figure 3.13). Both were active as observed from Disc diffusion test results. However, other strains grown in shake flasks did not show any activity after the supplementation of cell-free supernatant from corresponding biofilm cultures. Planktonic suspension cultures of *Pantoea* sp., SC-AF produced compounds with jelly-like appearance and made the culture viscous. Apart from that, SC-AF also produced a lot of foam (Figure 3.14) and was then selected for further analysis of polysaccharide production.

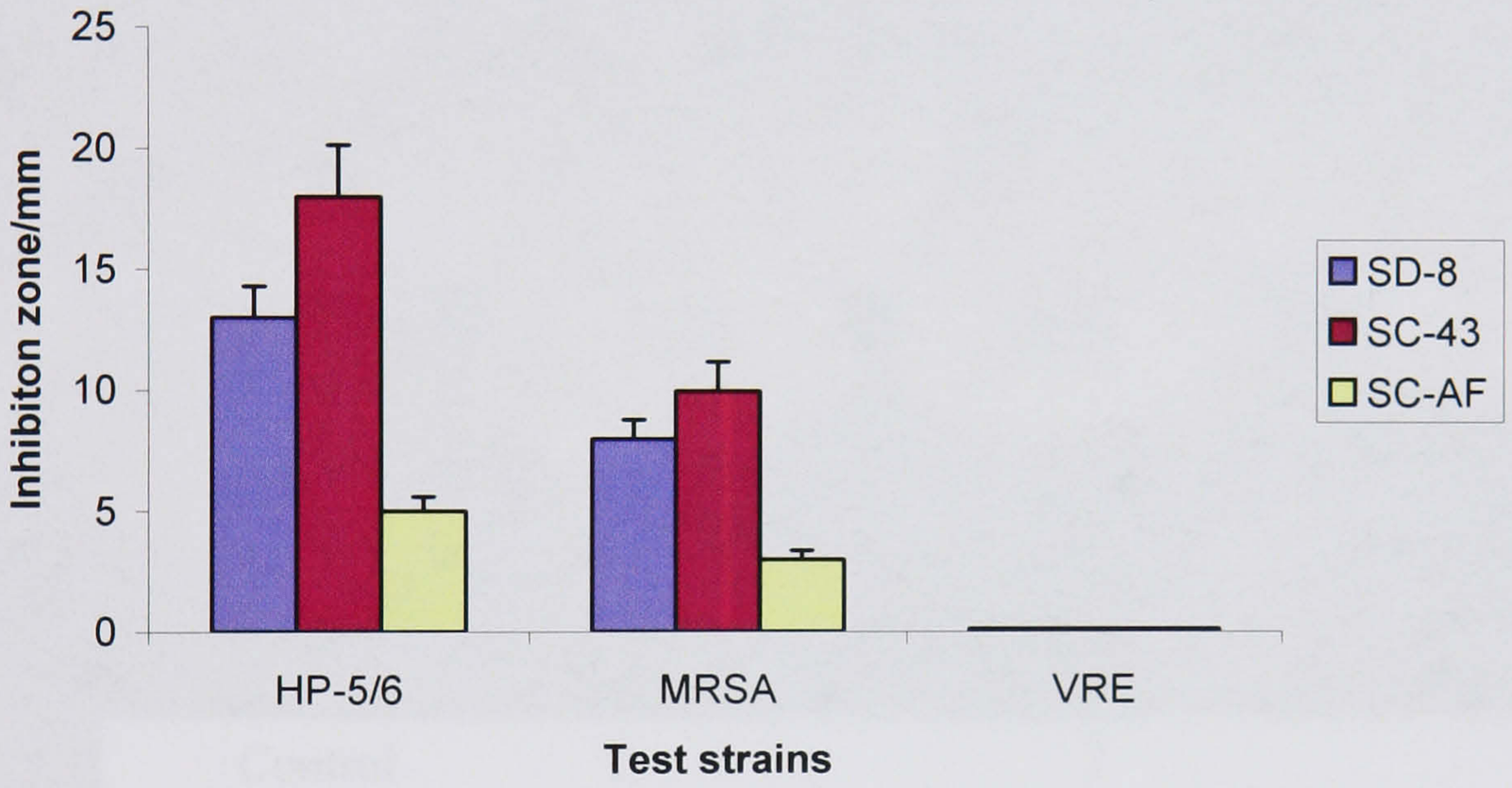


Figure 3.13 The induction effect of cell free supernatant from AMS cultivation. Cell free supernatant of AMS cultures of the same strains were added to the shake flask cultures of the same strains and cultivated for another two days. All presented antimicrobial activity against MRSA and *M. luteus*, HP-5/6. The cell-free supernatant from AMS culture could elicit the production of antimicrobial compounds.

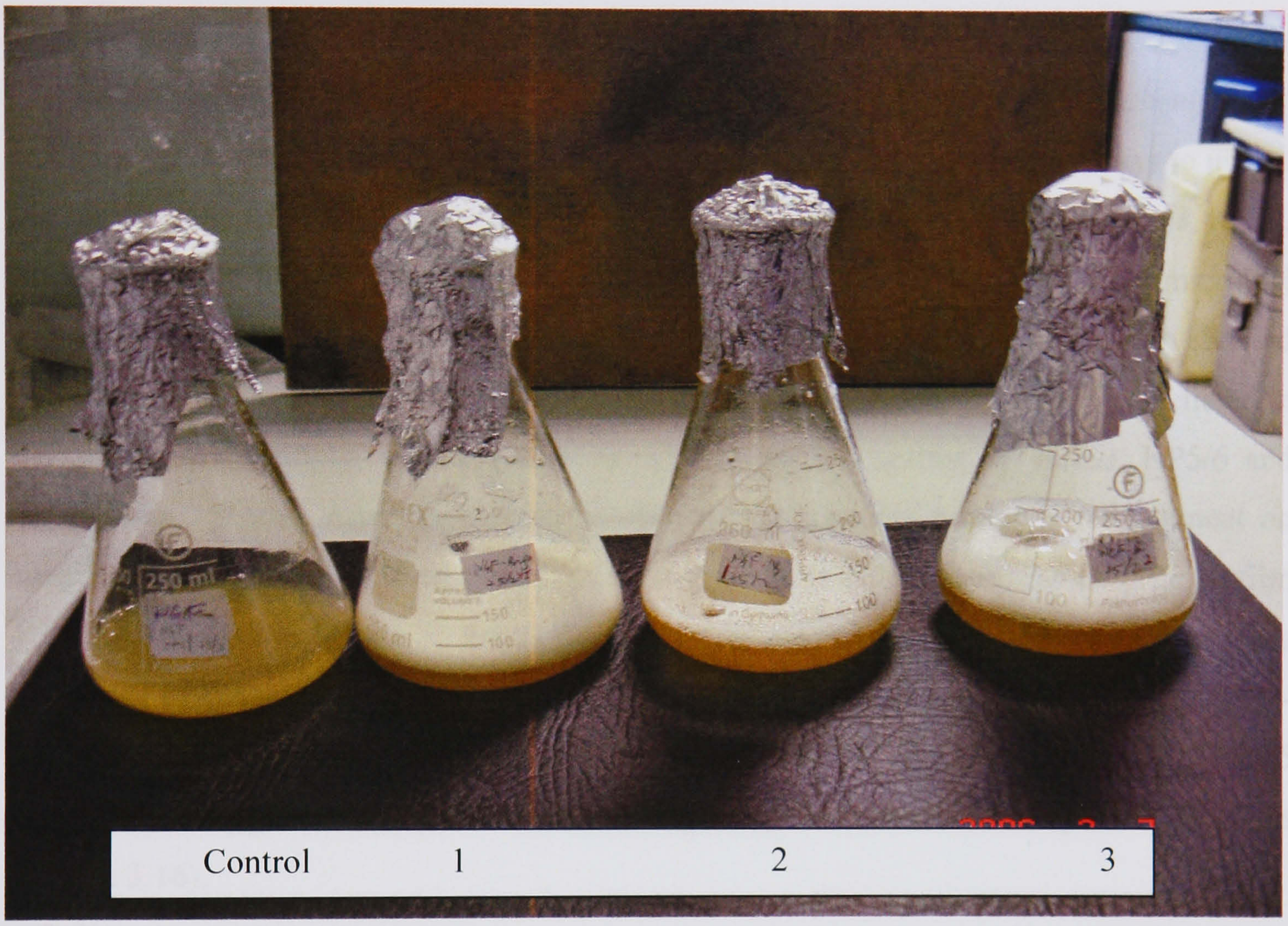


Figure 3.14 Shake flask cultures of SC-AF. Flask 1, 2, 3 were triplicates of strain SC-AF grown using 25 ml of NGF medium. SC-AF produced foam in NGF medium. Control: Nutrient broth used as a medium.

3.2.2 Isolates able to produce antimicrobial compounds when grown in biofilms in contact with the air

3.2.2.1 Air-membrane surface (AMS) cultivation

Five of the strains, *B. cereus*, HP22, *B. pumilus*, HP-43, B. subtilis, SD-8, *B. licheniformis*, SC-43 and *Pantoea* sp., SC-AF, were then cultivated in marine broth for five days to use on the membrane of the AMS culture. Several media were used (MB, SYZ, NGF, NB, ASW, NSW) in order to optimize nutrient conditions needed for production of antimicrobial compounds. There were only two culture supernatants from SYZ and NGF, which showed activities using the disc diffusion test. Strain names and antimicrobial activity are listed in Table 3.7. Interestingly, all the strains tested were active against *M. luteus*, HP5/6 and *Bacillus* sp., SD-28. Apart from that, SC-43 and SD-8 strains produced red pigment on membrane surfaces in AMS culture but not in shaken flask cultures. SC-AF built up biofilm with a jelly-like appearance on the membrane but no colour changes were observed (Figure 3.15). All of the cell-free supernatant (CFS) from AMS cultures of SC-AF, SC-43 and SD-8 still showed activity against target strains including MRSA after treating at 121°C for 15 min, which suggested that these antimicrobial compounds were unlikely to be proteins (Figure 3.16).

3.2.2.2 Scale-up pilot study using the ReacSyn®-Bioreactor

The ReacSyn®-Bioreactor was especially designed to grow biofilms in a pilot scale. Five strains, *B. cereus*, HP-22, *B. pumilus*, HP-43, *B. subtilis*, SD-8, *B. licheniformis*, SC-43, and *Pantoea* sp., SC-AF, were then used to scale up the production of antimicrobial compounds. Several types of membranes were used (PP-25, PP-1: Polypropylene membrane with pore size of 25 μm and 1 μm, NY5-HC, NY5-HD, NY1-HD: Nylon membrane (charged), NC: Nitrocellulose, Nylon: Nylon membrane (uncharged)) on the cartridge of the ReacSyn®-Bioreactor to optimise the production of antimicrobial compounds. Results indicated that the Nylon membrane was the best membrane for the ReacSyn®-Bioreactor to enhance the production of antimicrobial compounds.

Table 3.7 Five antimicrobial producing strains and media used in AMS cultivation

Strain name	Media used[1]	Antimicrobial activity against each other (Diameter of inhibition zone/mm)					
		[2]HP-5/6	HP-43	SD-28	HP-22	SC-43	SC-AF
[2]HP-43	SYZ	1	0	1	0	0	0
	NGF	1	0	1	0	0	0
SD-8	SYZ	1	0	1	0	0	0
	NGF	2	0	2	0	0	0
HP-22	SYZ	2	0	2	0	0	0
	NGF	3	0	3	0	0	0
SC-43	SYZ	2	0	1	0	0	0
	NGF	8	0	1	0	0	0
SC-AF	SYZ	1	0	1	0	0	0
	NGF	3	0	3	0	0	0

[1]SYZ: Soluble starch-yeast extract-NZ amine-dextrose medium, NGF: Nutrient-Glycerol-Ferric medium

[2]HP-43: *B. pumilus*, SD-8: *B. subtilis*, HP-22: *B. cereus*, SC-43: *B. licheniformis*, SC-AF: *Pantoea* sp., HP 5/6: *M. luteus*, SD-28: *Bacillus* sp.

(a)

(b)

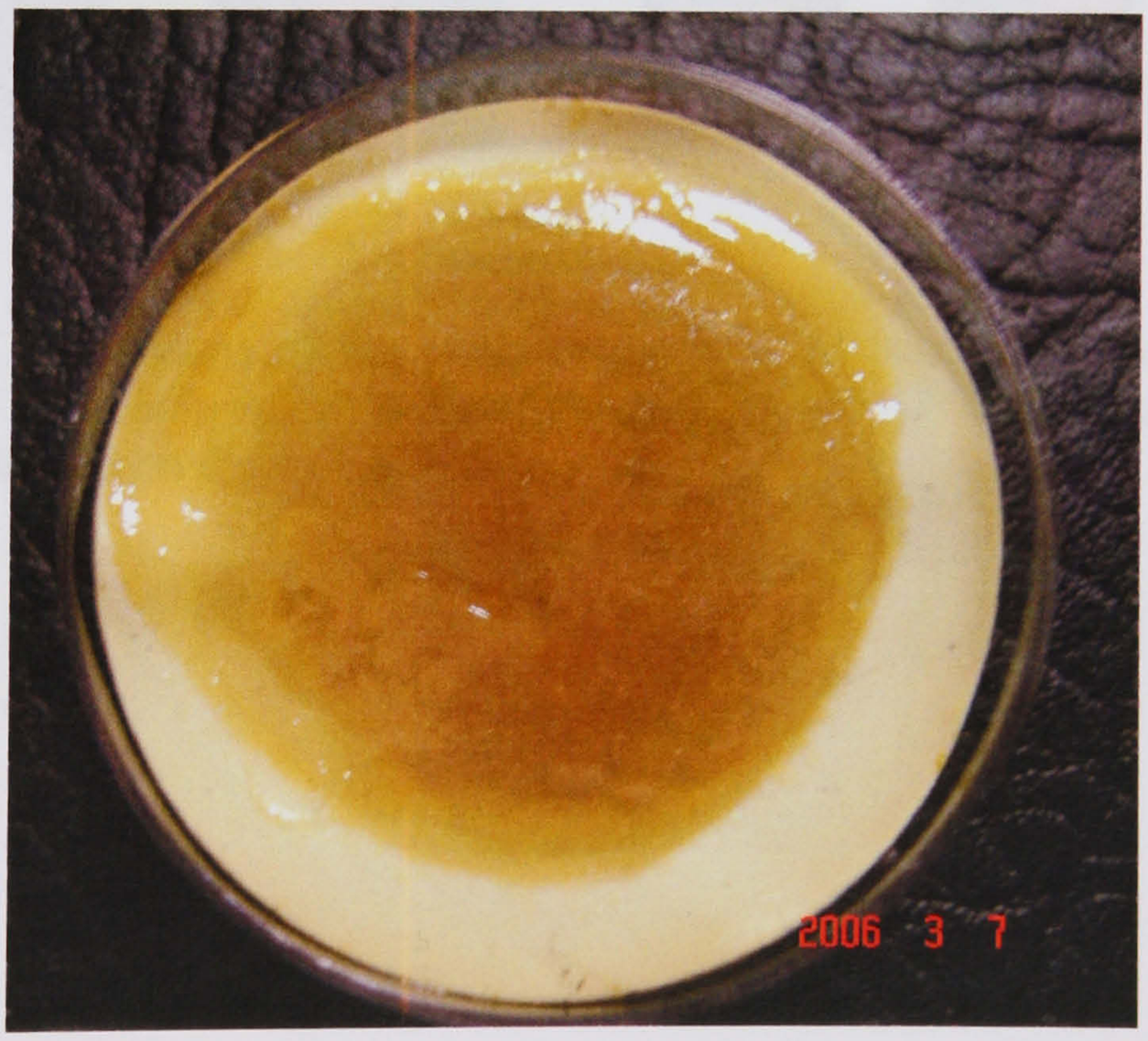


Figure 3.15 SC-AF producing biofilm with jelly-like appearance on membrane of AMS culture using (a) NGF medium: (Nutrient-Glycerol-Ferric) (b) Medium A-2: 0.2% Casein hydrolysate, 3% TSB, 0.2% yeast extract, 70% natural sea water.

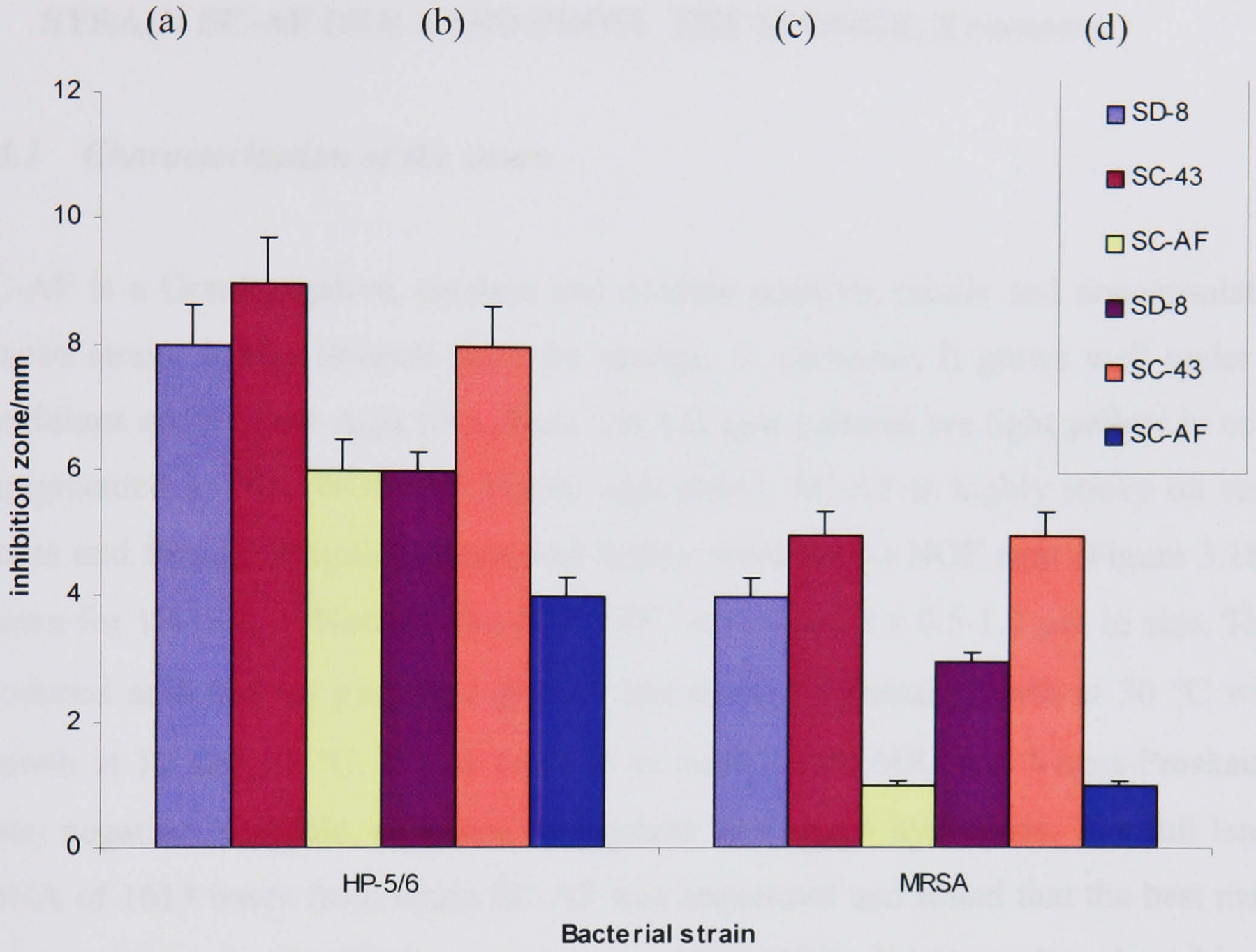


Figure 3.16 Comparisons of the inhibitory activity of bacterial strains, *B. subtilis* SD-8, *B. licheniformis* SC-43 and *Pantoea* sp. SC-AF (a) before and (b) after heat treatment on their CFS.

Selection of media from three types, namely SYZ, NGF and ASW, indicated that NGF was the best for the production of antimicrobial compounds. Results obtained from the ReacSyn®-Bioreactor are shown in Figure 3.17.

3.3 STUDY OF THE PRODUCTION OF ANTIMICROBIAL COMPOUNDS BY STRAIN SC-AF ISOLATED FROM THE SPONGE, *S.carnosus*

3.3.1 Characterization of the strain

SC-AF is a Gram-negative, catalase and oxidase positive, motile and noncapsulated, rod-shaped strain. It was isolated from the sponge, *S. carnosus*. It grows well under aerobic conditions on Nutrient Agar (NA) base. On LB agar cultures are light yellow in colour but unpigmented on NA, NGF and Marine agar (MA). SC-AF is highly sticky on semi-solid media and formed irregular shapes and highly wrinkled on NGF agar (Figure 3.18). Cells grown for 12-18 h in Nutrient Broth at 30°C were 0.3-0.5 x 0.5-1.0 μm in size. The strain produced acid and no gas from glucose and showed optimal growth at 30 °C with poor growth at 10 and 37 °C. It was positive in methyl red (MR) and Voges-Proskauer (VP) tests; negative in indole, cellulose, and gelatin and starch hydrolysis. The full length 16S rDNA of 1613 bases from strain SC-AF was sequenced and found that the best match was *Pantoea ananatis* (GenBank accession no. AY530798). However, based on biochemical results, SC-AF characteristics are different from *P. ananatis*. SC-AF was tentatively identified as *Pantoea* sp.

3.3.2 Optimization of culturing condition for antimicrobial production

From 3.2.2.2 (ReacSyn®-Bioreactor) and 3.2.1 (Shake-flask culture), none of the culturing condition could promote or elicit the production of antimicrobial compounds from *Pantoea* sp., SC-AF. However, SC-AF produced antimicrobials only when grown using AMS culture. Based on that, more than ten media were used to optimise the culturing condition for production of antimicrobial compounds by SC-AF.

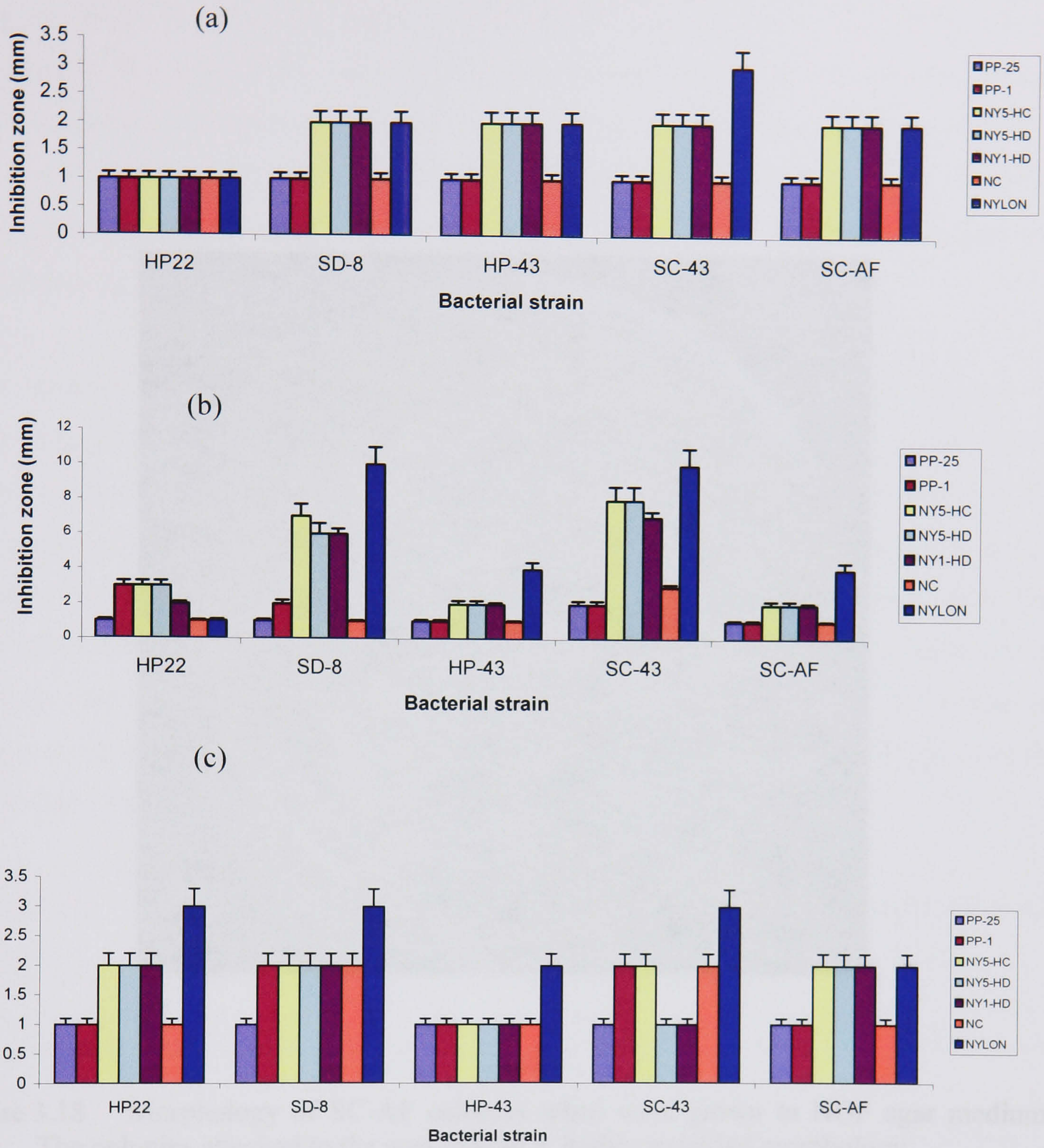


Figure 3.17 Comparisons of five antimicrobial compound producing strains grown in various media using ReacSyn®-Bioreactor. Media used a) SYZ, b) NGF, c) ASW. Five strains were *B. cereus,* HP22:,: *B. subtilis,* SD8,: *B. pumilus*, HP43, *B. licheniformis*, SC43, *Pantoea* sp., SC-AF. Different membrane types applied: PP-25: polypropylene-25μm, PP-1: polypropylene-1μm, NY5-HD, NY5-HC, NY1-HD: Nylon membrane (charged) but with different pore size (not specified by ReacSyn®-Bioreactor); NC: Nitrocellulose membrane, Nylon: Nylon membrane, (uncharged). The results suggest that nylon membrane (uncharged) is the best membrane for ReacSyn®-Bioreactor to enhance the production of antimicrobial compounds. NGF proved to be the best media for the production of antimicrobial compounds. HP 5/6 was used as test strains to indicate antimicrobial lawn.

Figure 3.18 Morphology of SC-AF colonies when were grown in NGF agar medium. The colonies attached to the agar exhibit a highly wrinkled morphology.

Only in NGF (Nutrient-Glycerol-Ferric medium) and Medium A-2 (0.2% Casein hydrolysate, 3% TSB, 0.2% yeast extract, 70% Natural sea water) did SC-AF produce antimicrobial activity against *M. luteus* (HP-5/6). The antimicrobial spectrum of SC-AF when grown using AMS culture in Medium A-2 supplemented with glycerol, NH_3NO_3, $FeSO_4.7H_2O$,$MnSO_4.4H_2O$, $NaNO_3$, and Menadione (100 μg/ml) did not produce any compounds as NGF or Medium A-2.

This indicated that only Ferric ion in the Nutrient Broth with glycerol elicited the production of the compounds. As for Medium A-2, it seemed that yeast extract and casein hydrolysates were needed for the production of the antimicrobial compounds from SC-AF. Since the activity was only moderate from both of the media (from Table 3.8), a new approach was introduced by using semi-solid medium (agar) instead of broth. After five days of incubation, agar media was punched and soaked in acetone. However there was no activity observed. The use of Medium A-2 and NGF broth medium for producing antimicrobial compounds by SC-AF has proven successful. Therefore, NGF was used for the further scale-up cultivation to obtain adequate antimicrobial compounds.

Table 3.8 Antimicrobial activity of strain SC-AF using two different media, NGF and Medium A-2

Target strain	Inhibition zone (mm/30 μl)	
	Medium used	
	NGF	Medium A-2
MRSA	2	1
VRE	0	0
HP-5/6	5	2
E. coli	0	0

3.3.3 Characterization of antimicrobial compounds produced using AMS

3.3.3.1 Molecular weight study

The fractions with molecular weights of less than 5 kDa showed more activity on disc diffusion tests compared to the fractions with molecular weight of more than 5 kDa (Figure 3.19). Out of three CFS samples (*B. subtilis*, SD-8, *B. licheniformis*, SC-43 and *Pantoea* sp., SC-AF); only SC-43 showed activity from both fractions. However, all the samples with However, all the samples with molecular weight less than 5 kDa showed stronger activity. Then, SC-43 and SD-8 were used as controls for further study of *Pantoea* sp., SC-AF.

3.3.3.2 Polarity study

The active compounds produced by SC-AF were not able to be extracted from culture supernatant using ethanol or butanol, which suggested that the compounds were polar. Of three solvents used to identify the polarity of the SC-AF compounds, methanol (MeOH) had the highest solubility compared with butanol and ethyl acetate (Table 3.9). Therefore, compounds produced by SC-AF were rather polar. However, methanol was able to dissolve the active compounds from the dried supernatant. Therefore, further purification was carried out using C18 reverse phase chromatography with H_2O-MeOH solvent gradient.

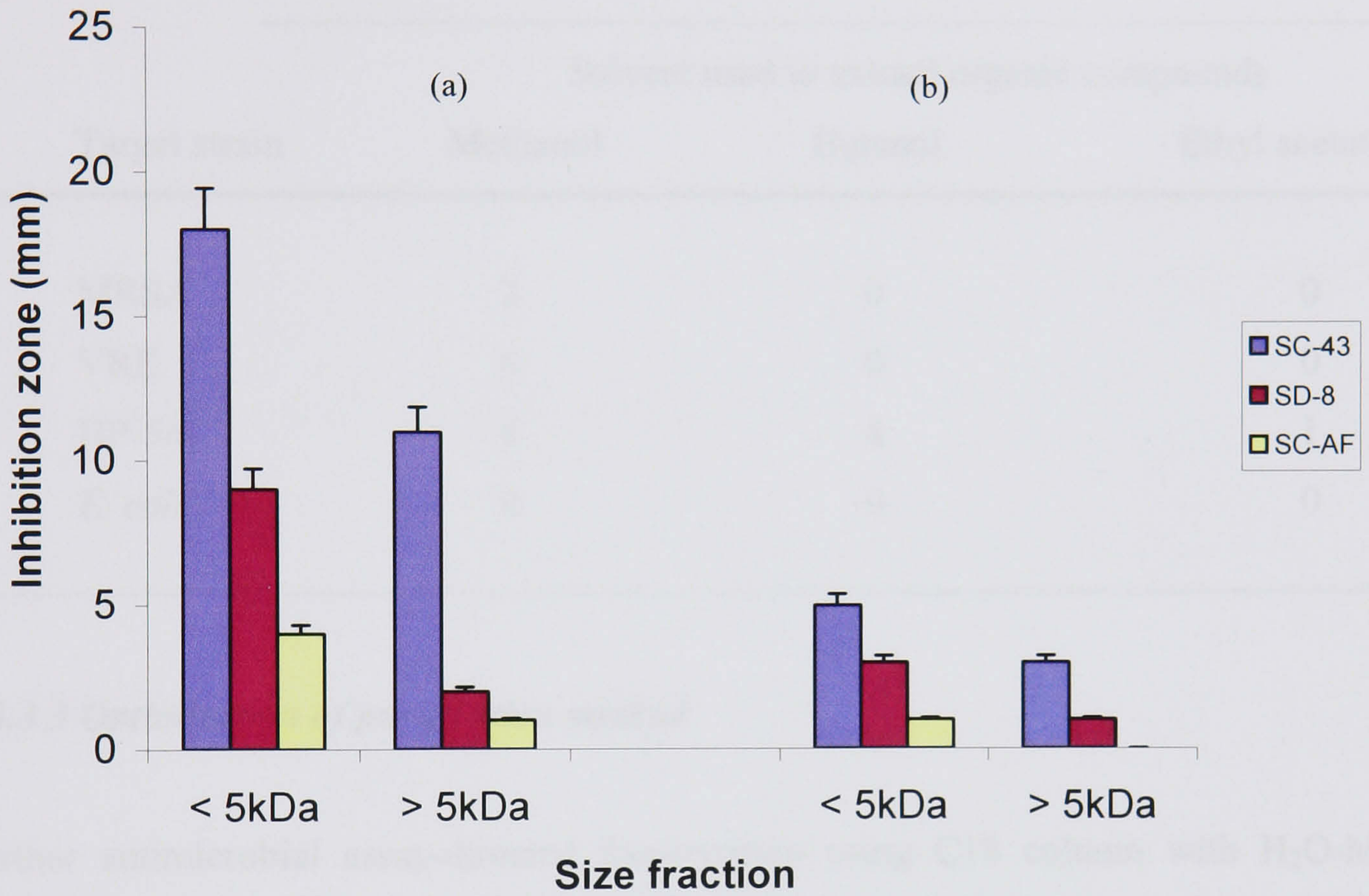


Figure 3.19 Inhibition zone of two fractions (molecular weight less than 5 kDa and molecular weight more than 5 kDa) of ultrafiltered supernatant from three strains, SC-43, SD-8 and SC-AF. Indicator lawn: (a) HP-5/6 (b) MRSA

Table 3.9 Antimicrobial activity of strain SC-AF using different solvents

	Inhibition zone (mm/30 µl)		
	Solvent used to extract organic compounds		
Target strain	Methanol	Butanol	Ethyl acetate
MRSA	2	0	0
VRE	0	0	0
HP-5/6	6	4	1
E. coli	0	0	0

3.3.3.3 Optimization of purification method

Further antimicrobial assay-directed fractionation using C18 column with H_2O-MeOH solvent gradient was carried out (Figure 3.20). Experiments indicated that only two fractions, 60% and 70% of H_2O used as a mobile phase, could elute the active compounds. Figure 3.20 indicates the activity of compounds on the disc diffusion test. The active fraction eluted from C18 column was further fractionated using HPLC system with H_2O-MeOH gradient indicating that the active fractions were eluted off at retention times of 36 + 37 min and 38 + 39 + 40 min (36%-40% MeOH concentration correspondingly, Figure 3.21). The figure shows the six different peaks whose correspondent fractions exhibited antimicrobial activity against a *M. luteus*, HP-5/6 strain (Figure 3.22). Each peak (1-6) was considered as a single pure compound; therefore strain SC-AF produced at least six different antimicrobial compounds. I have obtained between 0.8 mg to 1.1 mg of mass from each of the peaks.

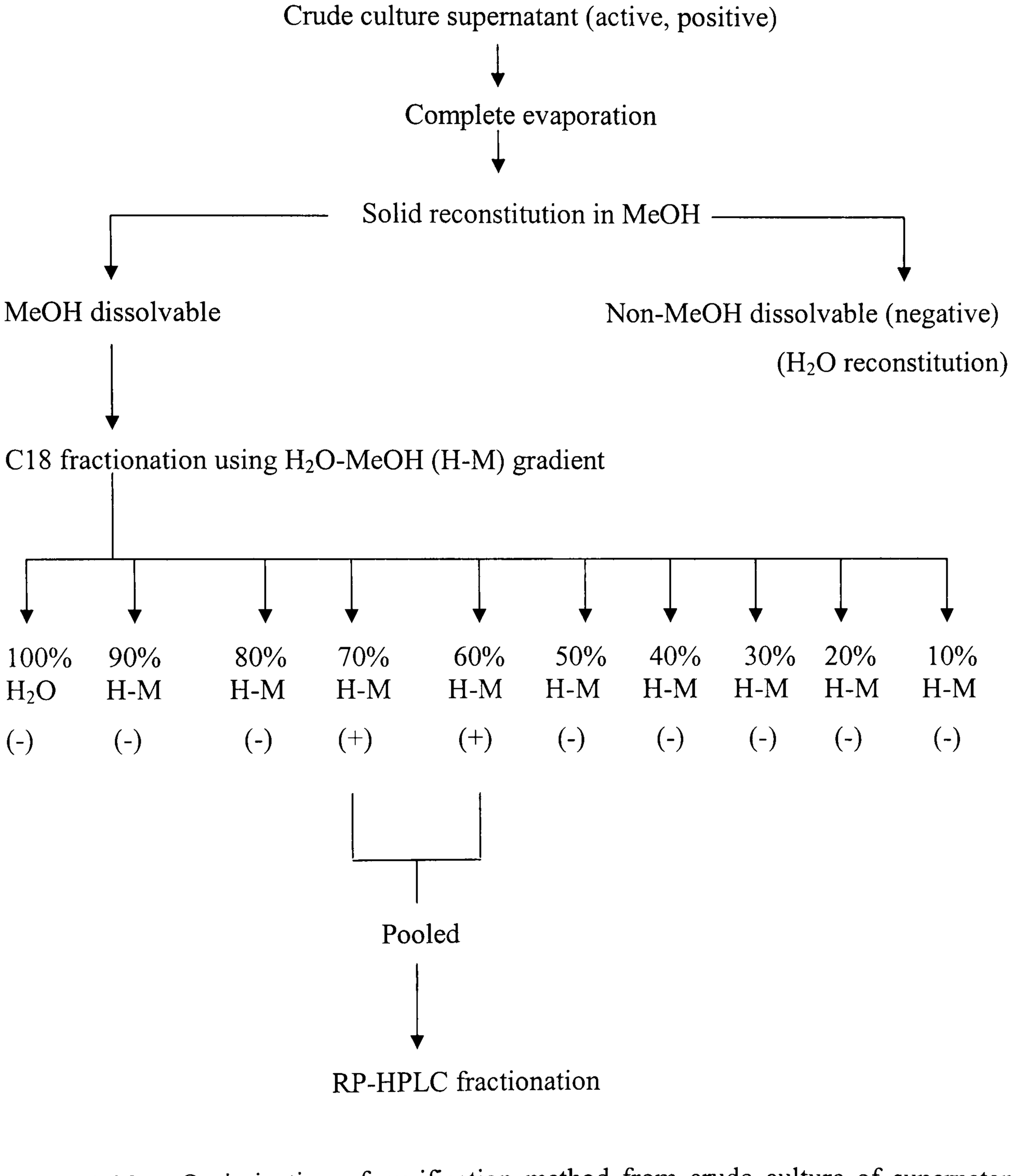


Figure 3.20 Optimization of purification method from crude culture of supernatant of *Pantoea* sp., SC-AF.

3.4 POLYSACCHARIDES PRODUCTION BY *Pantoea* sp., SC-AF

Results indicated a profile of polysaccharides accounting for the jelly appearance of the SC-AF biofilm. Fructose and cellobiose were characterized in their monosaccharide component. The polysaccharides also accounted for the thick supernatants in shake flask cultures. Figure 3.23 showed clearly the profile of sugars from SC-AF.

3.5 PRELIMINARY STUDY OF PROTON (^{1}H) NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR) AND MASS SPECTROMETRY (MS)

A partially purified approximately 1mg quantity of antimicrobial compound corresponding to peak 3 in HPLC fractionation was reconstituted in D_2O. Preliminary ^{1}H-NMR was carried out in a 200MHz magnetic field and the signal was accumulated for 32 min to obtain a basic spectrum to examine purity and possible candidate compounds. The spectrum suggests a great deal of impurities present in the sample. In addition, the amount of pure antimicrobial compound was also much below the sensitivity, thus it was very difficult to determine the structure. However, because there was no distinctive peaks in low field (left side) with high chemical shift (>7.5ppm), which could result from proton signal of amide in peptides, the possibility of the active compounds being a peptide can be excluded.

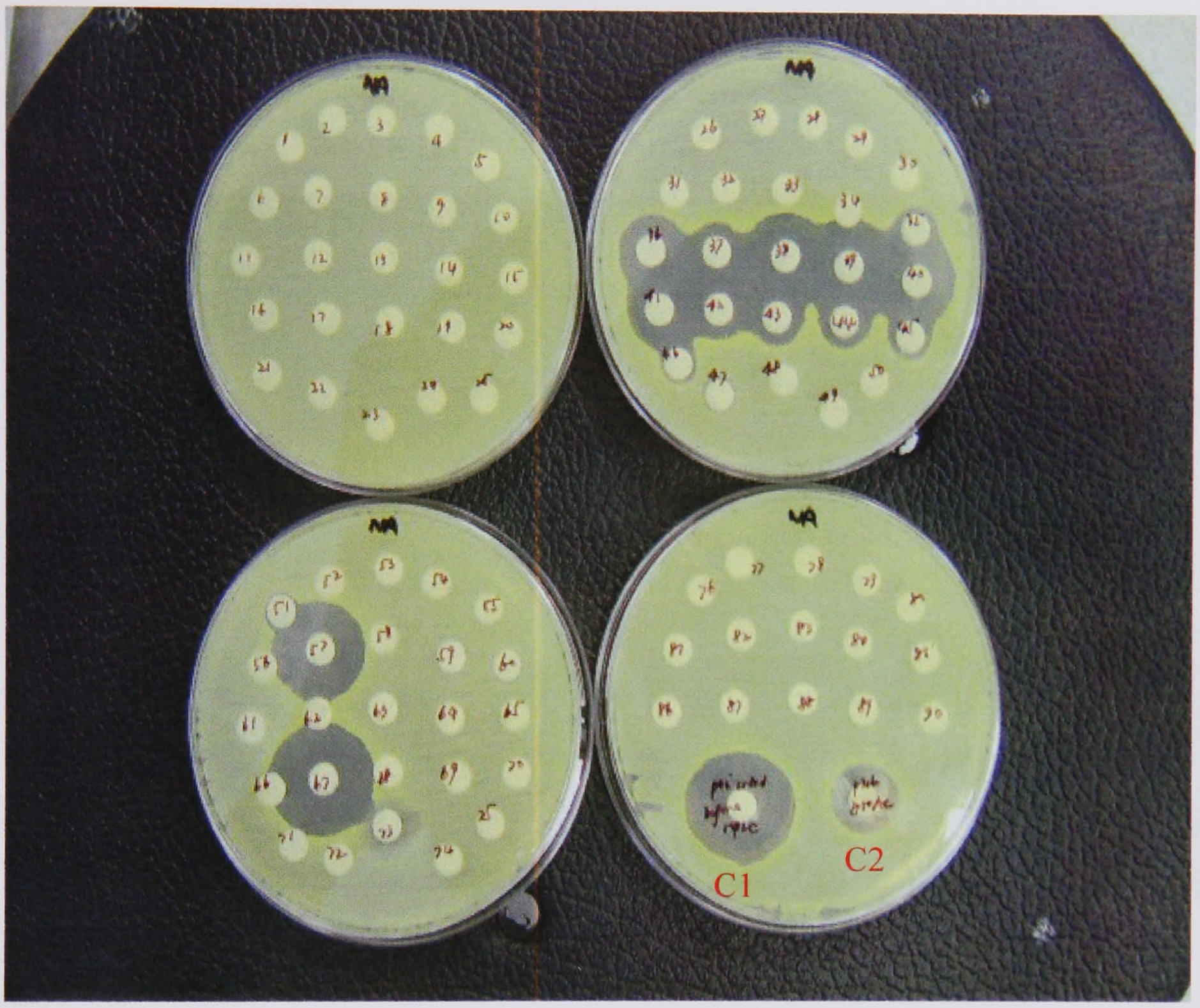


Figure 3.21 Determination of active fractions after C18-HPLC purification. The figure shows fraction 35, 36, 37 belonging to an active compound group. Fraction 38, 39, 40, 41 gave major activity and belong to the other active compound group. Activity was also observed at fraction 57 and 67 with 57 and 67 min retention time, respectively. It suggests at least four different active compounds. C1: First positive control before HPLC. C2: Crude AMS culture supernatant.

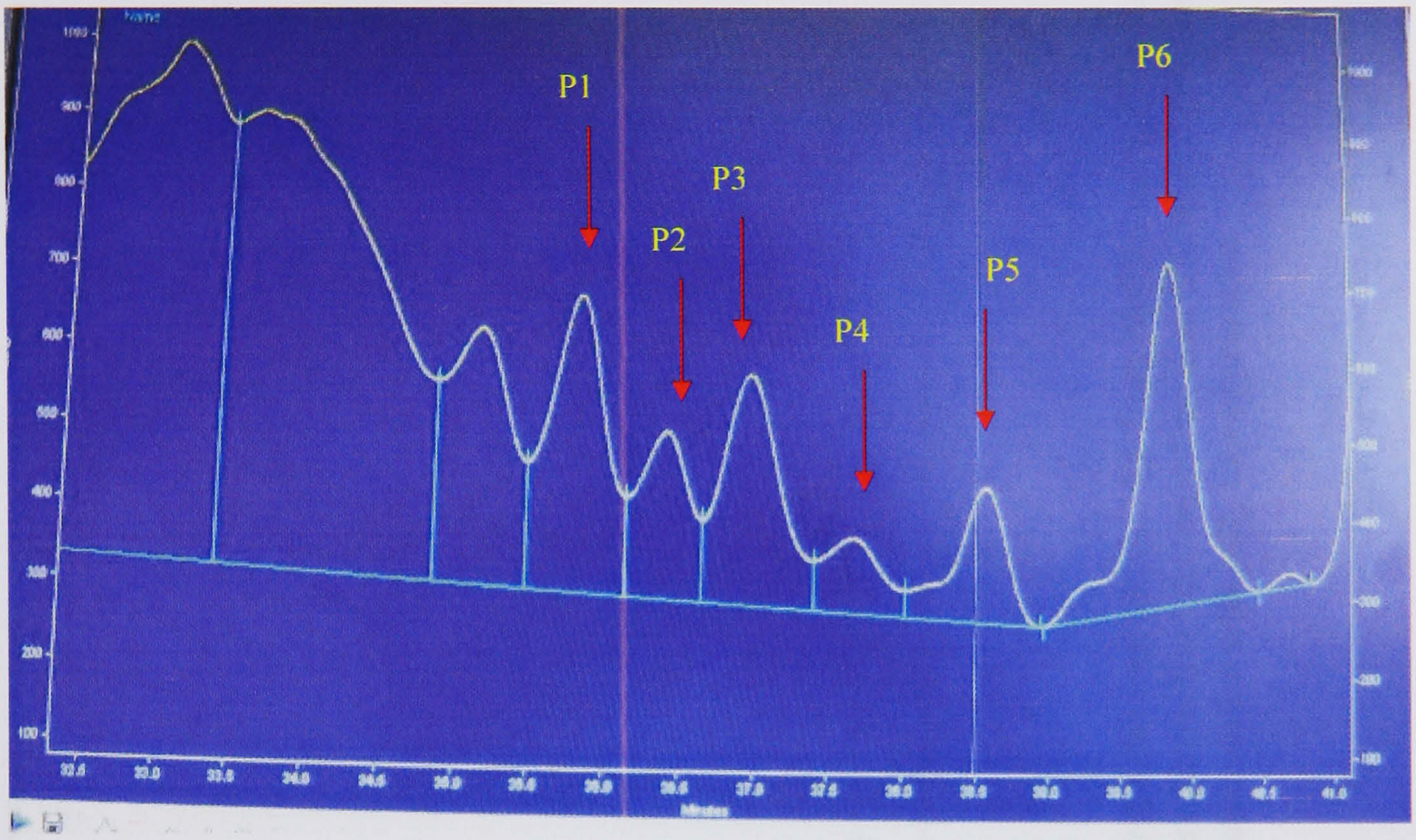


Figure 3.22 HPLC of SC-AF compounds with the main peak appears 36 + 37 min and 38 + 39 + 40 min after injection of the sample. The arrows indicate the active peaks of the sample.

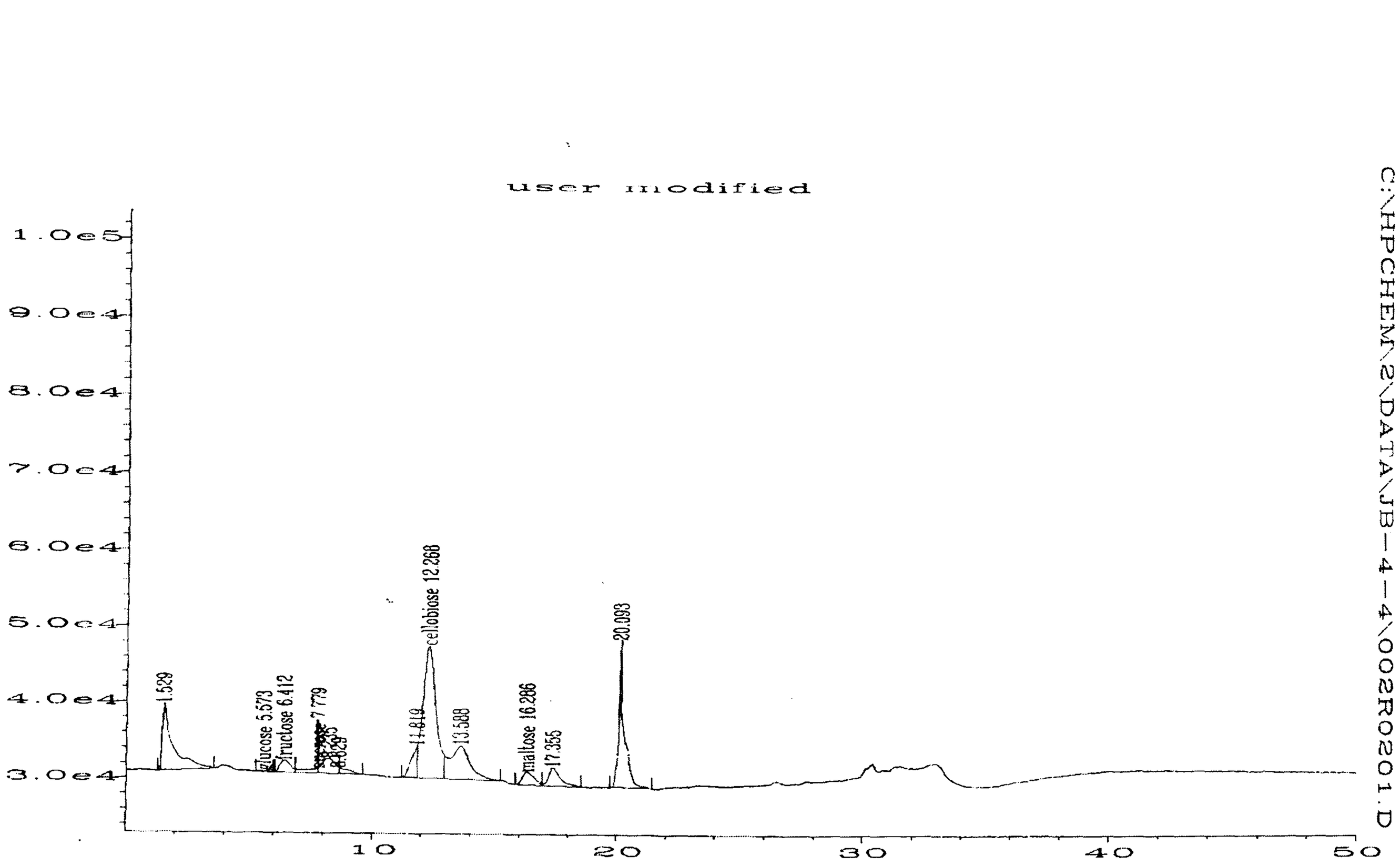


Figure 3.23 Profile of carbohydrates hydrolysed *Pantoea* sp., SC-AF from biofilm using Dionex Carbopac PA-100 Guard HPLC column. The figure shows fructose (retention time 6.514) and cellobiose (retention time 12.268) are the major unit of polysaccharides with jelly like appearance.

Therefore, it is confirmed that this compound is different from Pantocin A or B, whose structure have been characterised as peptides. The possibility of a fatty acid or sugar might be high (Fig. 3.24).

Results from Mass spectrometry (MS) showed that the major mass charge ratio (m/z) of base peaks of fraction 1 (Fr-1) and fraction-2 (Fr-2) are 385.4 and 423.4 (Fig. 3.25). Since the reported Pantocin B molecular weight is 296, Fr-1 and Fr-2 are antimicrobial compounds different from Pantocin B.

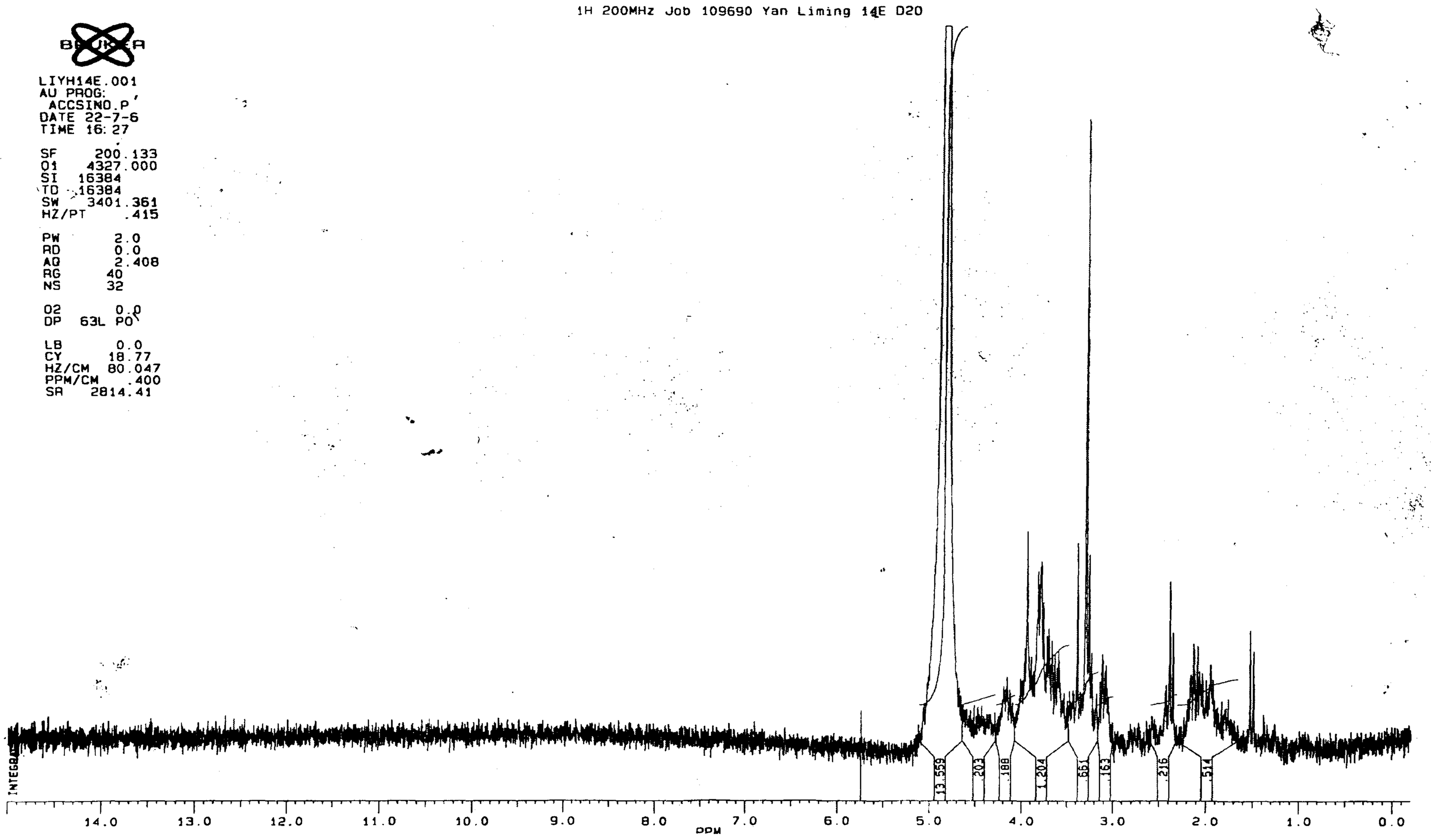
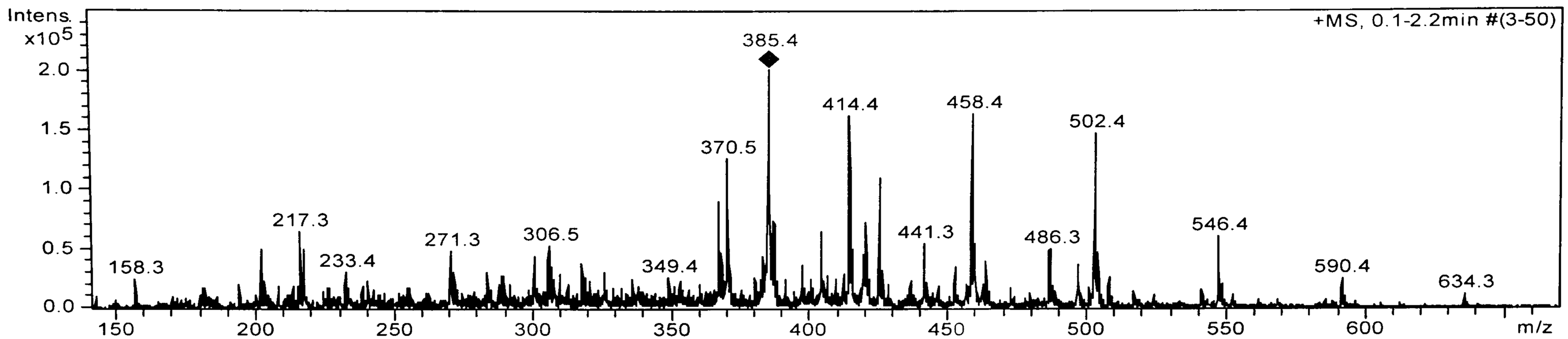


Fig. 3.24 Preliminary ^{1}H-NMR spectroscopy of purified antimicrobial compounds from *Pantoea* sp., SC-AF

Fr-1, target mass = *m/z* 300, positive mode, file SW000232.d:



Fr-2, target mass = *m/z* 300, positive mode, file SW000231.d:

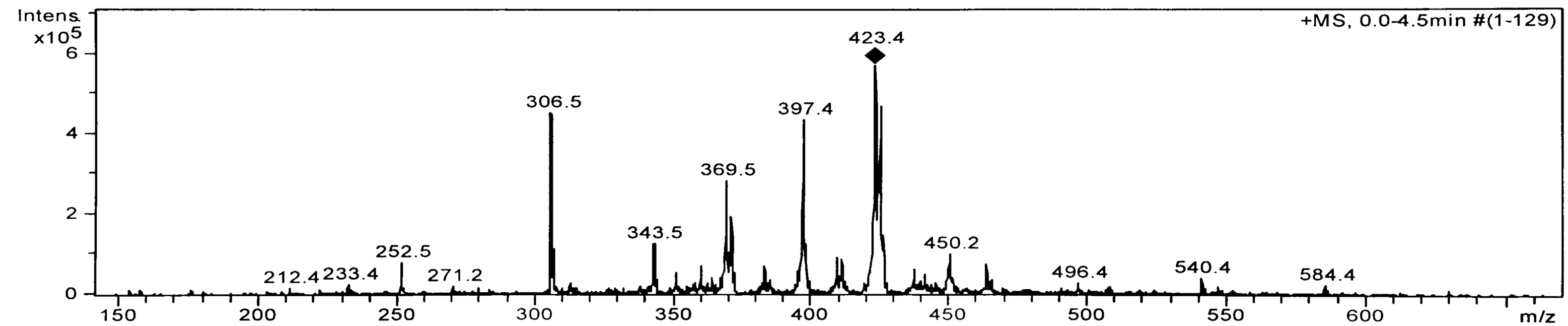


Figure 3.25 Mass spectrometry of fractionated antimicrobial compounds Fr-1 and Fr-2, from *Pantoea* sp., SC-AF.

CHAPTER 4 DISCUSSION

CHAPTER 4 DISCUSSION

4.1 DIVERSITY OF BACTERIA ASSOCIATED WITH SPONGES

4.1.1 Bacterial community characterized using cultivation-dependent method

Two aspects of sponge-associated bacteria have been studied in this research project. The first part is on the diversity of bacteria associated with the sponges; the second part on the bioactive compounds produced by sponge-associated bacteria, especially from a *Pantoea* sp., strain SC-AF. Prior to this study, a variety of sponge samples were used to identify and characterize the diversity of bacteria associated with sponges in order to get a maximum number of bacterial isolates. In this context, it is often desirable to analyse and compare a number of samples. Furthermore, use of a variety of sponge samples will help retrieve as many bacterial species as possible. Species identification and population organisation are critical in the study of microbial communities. Traditionally, microbial species are cultured and then characterized by their respective physiological and biochemical properties. Therefore, this approach will focus on culturable bacteria.

Prior to identification of unknown bacterial isolates, preliminary classification involved Gram staining (Hucker and Conn, 1923) and KOH lysis (Buck, 1982). Both methods can distinguish two groups of bacteria by the identification of differences in the structure of their cell walls. Generally, cell walls of Gram-positive bacteria are heavily concentrated with peptidoglycan with a single, porous, 20-80 nm layer of peptidoglycan (Alcamo, 1994). Gram-negative bacteria have considerably less peptidoglycan in their cell walls, with a 1-3 nm layer of highly porous peptidoglycan surrounded by a 7-8 nm outer membrane (Alcamo, 1994). Peptidoglycan, a complex carbohydrate, is thought to trap the crystal violet-iodine complex in its many cross-linkages, but the cell wall of Gram-negative bacteria would trap less of the complex crystal violet-iodine compared to the Gram-positive bacteria. Both methods can lead to equivocal results (Buck, 1982). In this study, 80% of the Gram-positive isolates identified by Gram-stain were virtually confirmed to be Gram-negative using the most reliable 16S rDNA sequencing analysis. Previous studies have shown that old age bacterial colonies or cells might give false Gram-negative results after

Gram stain (Gregerson, 1978). This results from the change of extra cellular matrix due to various reasons such as oxidation. Improper Gram-stained smears, such as making the smear too thick, excessive heat-fixing, and under or over decolorization may account for many Gram-stain errors. The high proportion of misidentified isolates due to procedural errors in carrying out the Gram stain cannot be wholly ruled out, nor can the possibility that Gram stains were performed on ageing cultures. This led to the fact that the majority of the isolates appeared to be Gram-positive by both staining methods, and 16S rDNA gene sequencing analysis later revealed these strains to be members of the genus *Vibrio* and *Pseudoalteromonas.* This suggests that a single method is not recommended for identification of isolates. In fact a previous report indicated that the KOH test incorrectly classified several anaerobic strains and suggested that the method should only be used in conjunction with the traditional Gram stain (Halebian *et al.*, 1981).

In this study I opted for the combination of culture-dependent methods and culture-independent methods (molecular methods). Results from the culture-dependent method indicated that more than 50% of the isolates obtained were Gram-negative bacteria. This finding was in agreement with previous studies where isolation of marine environmental microbes was often dominated by Gram-negative microorganisms (Zobell, 1946; Olson *et al.*, 2002). In fact a previous finding also indicated that only 1% of the bacterial isolates were from Gram-positive bacteria (Friedrich *et al.*, 1999). In addition the very limited number of Gram-positive isolates indirectly supported the previous finding that only 5% or less of the bacteria from sponges were culturable (Santavy *et al.*, 1990; Friedrich *et al.*, 1999; Olson *et al.*, 2002). Their findings suggested that the percentage of culturable bacteria recovered from marine samples can be higher than 0.1%, the recovery percentage which was reported from seawater. For example, 3.4 to 11% of the bacteria observed in association with the sclerosponge, *Ceratoporella nicholsoni,* were culturable (Santavy *et al.*, 1990).

The discussion of bacterial culturability may include the discrepancy between viable and direct counts. This may result from low plating efficiencies; thus only a few viable individuals in a population are capable of forming colonies on agar media (Rehnstam *et al.*, 1993). If living marine surfaces provide nutrient-rich environments, a relatively large

percentage of the bacteria associated with these surfaces may form colonies when inoculated onto nutrient-rich growth medium. Another focus is the inherent selectivity of all isolation methods. A limited group of bacteria will grow when one isolation method is used. In order to determine the percentage of culturable bacteria in any community, multiple isolation methods have to be attempted to determine the sum of all unique strains resulting from these methods (Jensen and Fenical, 1994). In this study, results indicated that, of all the identified strains from four species of sponges, *H. panicea*, *S. domuncula*, *S. carnosus* and *P. johntonia*, 44% were Gram-positive bacteria. The majority of the Gram-positive species obtained from this study were bacilli, including *B. subtilis*, *B. cereus, B. macroides, B. baekryungensis, B. firmus, B. pumilus, B. licheniformis, Paenibacillus* sp. and *Paenibacillus costantinii*. The genus *Bacillus* is ubiquitous in the terrestrial area, which has been exploited to produce various important industrial products.

It has been hypothesized that bacilli living in the marine environment are originally from the terrestrial area. In this study, a variety of bacilli were isolated from the sponges, and this genus dominated Gram-positive isolates. This suggests that bacilli might have particular symbiotic relationships with those sponges. Previous study has reported the same phenomenon from several marine organisms (Yan *et al.*, 2003). This could lead to a number of phenotype changes in bacilli. It has been noted that, *B. licheniformis* and *B. subtilis* produce red pigment when grown on NGF agar. In fact, the cells of *B. licheniformis*, SC-43 were very difficult to remove from the agar surface. This suggests that the strong attachment ability of this strain allow it to survive under strong current condition in the sponges, due to water pumping activities.

Bacilli have been demonstrated as a good source of useful compounds as well as showing other features of considerable practical importance, such as biocontrol agent (Felske *et al.*, 1998). Some thermophilic *Bacillus* species are crucial for biotechnological applications as they provide sources of thermostable enzymes and other products of industrial interest (Bergquist and Morgan, 1992; Felske *et al.*, 1998). Carboxymethylcellulase was also reported to be purified from a *Bacillus* sp. isolate associated with a marine sponge, *Axinella* sp. (Mohapatra, 1997).

It has been documented that *B. subtilis* and *B. cereus* play important roles as biological control agents of different phytopathogenic organisms (Emmert and Handelsman, 1999). Many members of the genus *Bacillus* are able to produce antibiotics. Interestingly, the majority of these antibiotics are low-molecular-weight peptides, which exhibit diverse biological activities, including antifungal, antibacterial, and antitumor activities (Hentschel *et al.*, 2001; Torres *et al.*, 2002). Most antibiotic peptides act on the bacterial cell membrane which will result in rapid damage to cell structure. This mechanism will give less chance for target strains to develop resistance, compared to other antibiotics which act on biochemical targets inside the cell.

Results from my study using culture-dependent method also showed that 90% of bacteria associated with the sponges *H. panicea*, *S. domuncula*, *S. carnosus* and *P. johnstonia* were species-specific. Approximately 10% of the isolates from four species of sponges were similar at the generic level. This indicated that low similarity of the strains was obtained at generic level. It may suggest that opportunist bacteria occurred transiently for food or happened to be in the water during the process of water pumping by the sponges (Imhoff and Stohr, 2003). Species-specificity of the bacterial isolates observed in my study suggests the presence of symbionts. My observation supported a few previous studies which have shown evidence for such a beneficial inter-relationship between bacteria and their sponge hosts (Burja *et al.*, 1999; Schmidt *et al.*, 2000; Hentschel and Steinert, 2001). An example in which a specific function of bacteria identified and localised within sponges may be the basis of a symbiotic inter-relationship. Other study reported that within the mesohyl of *Theonella swinhoei* distinctive filamentous bacteria occurred that were not found in other sponges from the same locations (Unson *et al.*, 1994). These bacteria identified as *Candidatus Entotheonella palauensis* produced theopalauamide for defense mechanisms of the host sponge (Schmidt *et al.*, 2000). Recent studies demonstrated that epibiotic bacteria on the surface of *S. domuncula* produce secondary metabolites that kill fouling bacteria, present in the surrounding of the sponge (Thakur *et al.*, 2003).

Results from my study also showed that the genus *Vibrio* was found associated with the sponges *H. panicea*, *S. domuncula* and *S. carnosus*; the genus *Paenibacillus* was associated with *S. domuncula* and *P. johnstonia*; the genus *Swanella* was associated with *H. panicea* and *P. johnstonia* and the genus *Kocuria* was associated with *H. panicea* and *S. domuncula.* This suggests that, although the majority of bacteria associated with sponges were species-specific, there were also a few non-species-specific bacteria associated with the sponges *H. panicea*, *S. domuncula, S. carnosus* and *P. johnstonia.*

Various growth media were chosen on the basis of their ability to support growth of a wide range of bacterial taxa. A variety of substrates such as Marine agar (MA), NGF agar (Nutrient-Glycerol-Ferric), YpSS agar, Nutrient Agar (NB) and Tryptic soya Agar (TSB) have been used. It is well established that growth media exert a dramatic selectivity on the recovery of cultured isolates (Suzuki and Giovannoni, 1996). In this study, after incubation at 27 °C for a week, plates were examined. The extended incubation time should ensure the recovery of slow growing isolates. Although the recovery of marine bacteria at 4 °C appeared to increase to certain extend, the growth rate became significantly slow (Yoko Okada, pers. comm.). MA was the best media for cultivation of isolates from the several media tested. In addition to peptone and yeast extract that provide good sources of carbon, nitrogen and vitamins, MA also contains minerals that nearly duplicate the major mineral composition of seawater. MA is a traditional nutrient-rich growth media, on the formulation for the isolation of heterotrophic marine bacteria with high salt content (Zobell, 1946). It helps simulate seawater and has been proven to be a good medium to grow marine bacterial isolates in the laboratory. MA is formulated according to the natural chemical composition of seawater. This suggests that high mineral content might help the recovery of marine bacteria from sponges. This is in agreement with a previous study showing that MA 2216E produced the greatest percentage and variety of antibiotic- producing bacteria from seaweed (Mearns-Spragg, 2000).

Culture-dependent methods do not accurately reflect the actual bacterial community structure, but rather the selectivity of growth media for certain bacteria. Furthermore, all techniques relying on cultivation are time consuming and expensive, as are the

physiological and biochemical differentiation tests (Theron and Cloete, 2000). Not only that, after the many generations necessary to form plate colonies, the organism may deviate from its physiology, and possibly even from the genotypic mix of the population in nature. Hence, a minor fraction (0.1 to 10%) of the bacteria can be cultivated using standard techniques (Brock, 1987). This is in agreement with this study that sponge associated bacteria identified from the culture-independent method were scarcely similar to the strains from the culture-dependent method.

My result confirms a previous study that bacterial species dominating a culturable assemblage were different from those identified with a constructed 16S rDNA gene library (Webster and Hill, 2001; Webster *et al.*, 2001a,b). These findings suggest that the diversity of sponge species, *H. panicea*, *S. domuncula, S. carnosus* and *P. johnstonia*, can be slightly over or underestimated depending on the populations present in an environmental sample. α-proteobacteria are commonly found associated with marine sponges and are often recovered in studies of sponge-microbial interactions.

In my study, apart from considerable isolates from α-proteobacteria, a high level of *Bacillus*-like sequences were also detected from all four sponge samples *H. panicea*, *S. domuncula, S. carnosus* and *P. johnstonia*. These results are in agreement with other findings (Pabel *et al.*, 2003). Because of the inherent selectivity of culture media and growth conditions, it is basically difficult to draw conclusions from culture experiments on the specific association of bacteria within sponges. In fact, sponges represent a special habitat for bacteria that is significantly different from the surrounding seawater and may include nutrient-rich and even microoxic niches. The different environmental conditions could give rise to specific but non-symbiotic associations of a commensal nature and could explain the dominance of different bacteria in and on sponges compared with the surrounding seawater.

Marine microorganisms may require special culture conditions to mimic their natural environment, such as high hydrostatic pressure in the case of deep-sea bacteria and an optimized production media for increased enzyme yield. They may also require an entirely different kind of complex nutrient in the production medium, which may be closer to the type of complex substances they are familiar with, unlike traditional sources such as soybean meal, molasses, or a chemically defined medium with known inducers (Chandrasekaran, 1997).

4.1.2 Bacterial community characterized using cultivation-independent method

Analysis of PCR amplified 16S rDNA fragments, combined with their separation on the basis of melting behaviour from environmental samples by DGGE, is a popular tool in microbial ecology (Muyzer *et al.*, 1993; Bruggemann *et al.*, 2000). This is a method by which fragments of DNA of the same length but different sequence can be resolved electrophoretically. In this study optimization of gradient range for the crude sponge extract showed that 40-55% of denaturant was the most reliable and had a high separation of PCR product. PCR-DGGE analysis indicated that a high diversity of bacteria was present in the four species of sponge, *H. panicea*, *S. domuncula, S. carnosus* and *P. johnstonia*. This result is in agreement with the previous study that showed DGGE was a good tool for fingerprinting of species diversity. It is relatively rapid to perform and many samples can be electrophoresed simultaneously (Theron and Cloete, 2000). Comparison between different sponge samples and surrounding seawater also provide information on the presence of a sponge-specific bacterial community.

S. domuncula and *P. johnstonia* were observed to have low species diversity. *S. domuncula* displayed only four to seven detectable bands, whereas *P. johnstonia* had four to nine. DGGE banding patterns of *S. domuncula* and *P. johnstonia* exhibited a very different bacterial community structure than the other two sponge samples, *H. panicea* and *S. carnosus*. The banding pattern of *S. domuncula* associated bacteria was at the top of the gels, suggesting that low G+C bacteria were enriched, because a DNA fragment with a high G+C nucleotide content denatures at a higher denaturant concentration than a molecule

with a low G+C content (Muyzer *et al.*, 1993). The highest species diversity was shown by *S. carnosus*, followed by *H. panicea*.

Sequence information from such a small rDNA fragments for DGGE cannot readily be used to compare populations, nor is it accurate to apply such short partial sequences to construct phylogenetic trees, especially if the sequences lack close relatives in the database (Kruse *et al.*, 1998). Furthermore, it has been shown that the PCR amplification of 16S rDNA fragments can be biased by several parameters (Reysenbach *et al.*, 1992; Suzuki and Giovannoni, 1996; Curtis and Craine, 1998). Most recently, it has been shown that PCR amplification of 16S rDNA was highly biased, so that the rDNA from one species was preferentially amplified (Hansen *et al.*, 1998). To overcome such bias, previous study has suggested the use of at least two different sets of primers (Hansen *et al.*, 1998). It has been further demonstrated that some primer pairs give a strong correlation between the ratio of the genes in the starting mix and the ratio in the final PCR product (Suzuki and Giovannoni, 1996).

At the early stage of PCR-DGGE experiments the set of primers used were 314F-GC and 518R. After a few attempts no sequences were successfully obtained, although PCR bands have been obtained successfully. Hence, a new set of primers 968F-GC – 1401R were used. It is highly recommended that at least two different primer sets be used to study the diversity of microbial communities by DGGE (Dahllof, 2002). Therefore I used a second set of primers 968F-GC – 1401R, and the corresponding DGGE profiles again showed that the genomic fingerprints of the bacterial communities of the bacteria associated with sponges were different. Only this set of primers, which generated a larger, 433-bp fragment, were used to determine the sequence of the resulting DGGE bands. I noticed that there was some limitation in the use of "single" DGGE band to carry out sequencing analysis, although many studies have used the same primers targeting the V2-V3 region of the 16S rDNA gene for identification of bacteria associated with sponges (Taylor *et al.*, 2004b).

The usage of primers with such a short size will lead to PCR artefacts because of highly folded loops and stem (Walter *et al.*, 2000). Perhaps non-specific target templates during PCR may lower the bacterial population and will lead to overestimation of the bacterial

content (Hansen *et al.*, 1998). One specific limitation of DGGE is that only short sequence fragments can be used (up to 500 bp), thereby limiting the amount of sequence information for subsequent identification by comparative sequence analysis. Bacteria may harbour more than one copy of the 16S rDNA encoding gene, with heterogenous sequences, giving rise to more than one band on DGGE (Nubel *et al.*, 1996; Nubel *et al.*, 1997). Furthermore, dissimilar sequences may co-migrate to the same position in a DGGE gradient (Rossello-Mora, 1999), causing a band to be a mixture of more than one sequence and preventing recovery of a clean sequence after re-amplification. Artificial bands may be due to heteroduplex molecules (Ferris and Ward, 1997), which may form between single strands or two similar, but not identical, DNA molecules. Similar "single" DGGE bands were not always found to represent a single bacterial strain (Sekiguchi *et al.*, 2001). The bands, which migrated to the same position in different lanes, may consist of different bacteria. They found that the heterogeneity was also ascribed to the presence of faint bands, which were located very close to or overlapping the target band. Therefore DGGE can be recommended for pattern analysis without further sequencing if a non-heterogeneous gene is used, such as 16S rDNA.

The DGGE patterns obtained for all samples showed high degrees of similarity from 16S rDNA amplified within the species of sponges, whereas significant differences were observed between the species of sponges. This suggested a host-specific bacterial community but this was very hard to verify since none of the sequence results were obtained. Traditionally, the distinctions of the bands on the DGGE gel and the determination of individual species concentrations rely on visual observation (Curtis and Craine, 1998). The technique becomes less reliable for samples of complex communities; bands of some species could be too close to be differentiated. Also, it may overlook species at low concentrations, which have low-intensity bands. To overcome the problem, a digitization method has been introduced (Zhang and Fang, 2000). By use of a sophisticated image analyzer, it is possible to detect all the major microbial species and quantify their relative concentration in one single analysis (Zhang and Fang, 2000).

In order to have an insight into the species richness of the microbial community of sponges, a cloning method was used. Results showed that bacterial isolates present in the sponges indicated different predominant groups as compared with the culture-dependent method. It suggested the presence of unculturable bacteria from the clone library obtained. The difference in detection and sensitivity was probably a result of PCR bias associated with the total DNA extracted from crude sample of sponges (Suzuki and Giovannoni, 1996). Numerous studies have shown species bias with different DNA extraction methods (Martin-Laurent *et al.*, 2001; Li and Liu, 2006). The extraction efficiency can be checked, although this is hardly feasible when working with a large number of samples. It is therefore important to choose the right extraction method for the community in question. Bias associated with clone library construction and random selection of clones for restriction analysis will affect the representation of the microbial community (Liesack *et al.*, 1991).

In this study, most strains identified from culture-independent methods did not seem to have their counterpart isolates using culture-dependent methods, although the latter was supposed to be detected when using the former method. Interestingly, *Synechococcus* sp. (Cyanobacteria) obtained from this study was also reported in other sponge species (Hentschel *et al.*, 2002; Thacker and Starnes, 2004; Usher *et al.*, 2004; Steindler *et al.*, 2005) suggesting that species-specificity may not apply to all sponges, and this species might be easily captured by various sponges. It has been demonstrated that there is a group of symbionts comprising at least four closely related species of *Synechococcus*. It has been suggested that sponge-associated *Synechococcus* are genetically distinct from planktonic *Synechococcus* sp., but there was no evidence to show that these symbionts were host-species-specific (Usher *et al.*, 2004). In my study cloning results indicated that redundant sequences after screening might be inevitable. Earlier work showed the same phenomenon: among 500 clones with the 16S rDNA fragment, only about 150 different operational taxonomic units were identified (McGarvey *et al.*, 2004).

Other study has demonstrated that a combined clone and DGGE strategy can minimize redundant sequencing, although PCR products from excised bands had to be cloned to obtain pure DNA sequence (Burr, 2006). It has been further suggested that cloned PCR

products generated using different primers resulted in significantly different composition of clone libraries (Rainey *et al.*, 1994). Furthermore, this study found that the same batch of PCR product cloned using either blunt-end or sticky-end cloning procedures gave different results. In my study I did not observe results similar to a previous report that phenotypically similar bacteria were isolated from 9 of 10 sponges species collected from two distinct geographical regions (Wilkinson *et al.*, 1981). Therefore, my study was in agreement with previous report that non-species-specific bacterial association with sponges occurred (Hentschel *et al.*, 2002; Olson *et al.*, 2002). The clone libraries revealed the presence of diverse assemblages of bacteria in the microbial communities of all sponges studied.

The agreement between the methods using different primer sets indicates that PCR bias did not alter with microorganisms detected, but it did affect the frequency of detection. All methods provided a qualitative assessment of the enriched microbial communities, but were not effective for presumptive enumeration of species within the community, which would require alternative methods such as fluorescent *in situ* hybridization (FISH).

Phylogenetic trees demonstrated the existence of members of a variety of bacterial phyla in the four sponges I studied. This has been reported by other studies as well but varied between sponge sample and investigating procedure (Webster *et al.*, 2001b; Webb and Maas, 2002). In this study 16S rDNA gene sequencing was carried out to provide insights into the bacterial strains in sponges. BLASTN searches of partial sequences of each strain suggested that they consisted of a phylogenetically diverse group of bacteria (Fig. 3.4 and Fig. 3.5). The construction of phylogenetic trees of partial 16S rDNA gene sequences derived from Gram-negative and Gram-positive isolates confirmed this. Eight distinct genera were identified which suggested the diversity group of bacteria involved in the sponge. Despite this observation, phylogenetic tree of clones (Fig.3.11) indicated distinct genera compared to the previous phylogenetic tree (Fig. 3.4 and Fig. 3.5). A group of cyanobacteria and uncultured bacteria were obtained. This suggests that integrated methods will help to gather information on the microbial diversity on sponges.

A uniform microbial community was also discovered regardless of species of sponges or geographic regions (Hentschel *et al.*, 2002). These authors made a comparison of 16S rDNA sequences from five different sponges and hypothesized that sponges have a uniform microbial community irrespective of species and location. They found that the majority of sponge-derived sequences were related to *Acidobacteria*. In addition, phylogenetically similar strain has also been found to accommodate in different sponges in different location. For example, strain NW001, an α-proteobacterium which appeared to be specifically associated with a Great Barrier Reef sponge, *Rhopaloides odorabile*, also identified by others from different sponges closely related to NW001 (Olson *et al.*, 2002). NW001 was detected localized in choanocyte chambers of the mesohyl region by using FISH. Conversely, a similar α-proteobacterium, MBIC 3368 whose 16S rDNA sequence was identical to NW001, was unable to be localized in the mesohyl of sponge, *Aplysina* sp., through FISH analyses (Friedrich *et al.*, 1999; Hentschel *et al.*, 2001). However, in my study no *Acidobacteria* 16S rDNA sequences were detected. Here, a much larger number of Gram-positive bacteria, which have been identified as *Bacillus* sp., were isolated through the cultivation-dependent method, and *Synechococcus* sp. (Cyanobacteria) was identified from a constructed library of 16S rDNA gene clones. My findings can be supported by a study using fluorescent *in situ* hybridization (FISH).

Both of the strains are closely related, based on 16S rDNA sequence homology, however, the uniform microbial community was not observed. This suggests that sponge microbial communities are not as uniform as previously declared although it is difficult to compare different studies directly. It has been noticed that different DNA extraction and amplification procedures can carry their own biases and limitations. The only way to increase knowledge of sponge specific communities and possible symbiotic relationships is to increase the number of sponges studied in this way and over a continued long time range. Additional studies are worth carrying out to study *H. panicea, S. domuncula, S. carnosus* and *P. johnstonia* living in different biogeographic environments.

4.1.3 Comparison study of bacteria associated with S. carnosus in different environments

The results showed significant differences in the bacterial community associated with sponge samples from natural resources and an aquarium. This suggests that establishment of the bacterial community actually depends on the seawater as well as the surrounding environment. My results showed that the diversity of bacterial populations of the sponges, which were transferred from the natural marine environment to an aquarium, gradually decreased with time. I also found that a few predominant groups of bacteria were present both before aquarium culture and after six month of aquarium culture (Figure 3.12), which strongly suggested that there is some of the species-specificity in the associated community of *S. carnosus*. This has been reported by various studies from different of sponges (Wilkinson *et al.*, 1981; Hentschel *et al.*, 2002; Olson *et al.*, 2002). DGGE analysis also showed that the banding pattern generated from 'sponge tissue' is different than the banding pattern generated from seawater samples.

This result suggested the existence of symbiotic bacteria within the sponge, and was consistent with another previous study (Enticknap *et al.*, 2006). Some common bands, which were observed in the entire sample, might be due to the sponge's filter feeding activity. This could take place as a sponge filters the surrounding water using bacteria for food. By processing large volumes of seawater, marine sponges are also constantly exposed to a wide range of other microbes including potential pathogens in the surrounding environment that may influence the diversity of the sponge-bacteria association. The molecular techniques applied in this study indicated that there was a complex microbial community associated with the sponge.

This study seems to be the first attempt to investigate the kinetic change of sponge-associated bacterial communities using sponge samples transferred from the natural marine environment to an aquarium. The use of culture-independent methods to monitor the gradual change of dominant bacterial populations also provides a better strategy to learn more detail during the community change with time compared with culture-dependent

methods. There was a high level of similarity between the DGGE profiles obtained after PCR amplification of sponge 16S rDNA, regardless of the site and parts of the sponge.

The diversity of bacteria isolated from the sponges cultured in the aquarium seemed to be significantly different in term of detectable bands derived from partial 16S rDNA. Before the aquarium cultivation started, patterns of the DGGE bands seemed to be similar between the two sponge samples, except that the sample for aquarium cultivation contained a number of additional bands and a higher level of signal. However, after cultivation for six months, the DGGE profiles derived from the sponge in the aquarium decreased significantly, with many bands being absent. The missing bands could be due to the absence of the appropriate natural environment to maintain the growth of the bacteria within the host. This also suggested that these disappeared bacteria were not true symbionts but just associated.

Theoretically, the intensity of the PCR product was supposed to be proportional to the abundance of the template and therefore the abundance of each population (Muyzer *et al.*, 1993). However, many studies have found that the intensity of the bands did not necessarily reflect the concentration of the bacterial strains in the samples, but their ability to grow in the different sets of environments applied. A number of studies have reported success in using DGGE to distinguish bacterial strains among diverse species (Moeseneder *et al.*, 1999; Diez *et al.*, 2001; Enticknap *et al.*, 2006). Differences in band intensity which occur frequently, are extremely difficult to detect and can offer false results. Furthermore, a missing band or bacterium may not be absent from the sponge host but merely at a level below the detection limit (Taylor *et al.*, 2005).

In this study DGGE analysis also shows that the banding pattern generated from the sponge tissue was different from that generated from their surrounding seawater sample. The other bands that were not apparent in the water sample could be evidence of symbiotic bacteria within the sponge. The banding pattern from sponge tissue was more complex than that from the seawater sample, which suggested a much higher diversity and enrichment of bacteria. Further sequencing analysis of excised bands was not successful. However, these negative results did not suggest any faults in the experiment. Actually, the sequencing

analysis spectrum strongly suggested the heterogeneity of the DNA templates should account for the unreadable sequencing results (data not shown). Therefore, a single DGGE band did not necessarily suggest a pure DNA product.

In some other studies, the authors also reported that no DNA sequence data at all has been obtained following DGGE analysis (Zhang and Fang, 2000; Dilly *et al.*, 2004). Furthermore, excised bands provide limited sequence data (generally <500bp) (Muyzer, 1999; Yu and Morrison, 2004). Thus, in this study DGGE was therefore not conclusive in indicating the presence or absence of symbionts or similar bacteria in the water sample. In this study results demonstrated that DGGE can provide a profile of the whole community of sponges and can facilitate screening of large-scale samples. In conclusion, the DGGE method has provided a reasonably detailed view of marine sponges assemblage and allowed tentative phylogenetic identification of the dominant members. Although the results suggested that a highly diverse bacterial community is associated with sponges, these findings addressed the ecological question of whether bacterial populations observed in the community, based on phylogenetic analysis, are those metabolically active in these communities.

In particular, by means of the molecular approaches, it was found that a few sequences retrieved did not correspond to any previously recognised 16S rDNA sequences from culture-dependent method. This strongly suggests the presence of new, previously undescribed taxa. The application of techniques in molecular biology to the study of microbial ecology has confirmed previous beliefs that bacterial diversity in marine sponges is far greater than can be accounted for by the culture-dependent techniques typically used to describe bacterial communities.

4.2 SCREENING OF SPONGE ASSOCIATED BACTERIAL ISOLATES TO PRODUCE ANTIMICROBIAL COMPOUNDS

4.2.1 Antagonism between sponge-associated bacteria

My results in this study suggested bacteria associated with sponges do have potential as a source of antibiotics. Antimicrobial compounds from the supernatant of bacterial isolates have shown reasonable merit for further research to be carried out. In this study results indicated that *B. licheniformis*, SC-43, *B. subtilis*, SD-8, *B. pumilus*, HP-43 and *B. cereus*, HP-22 displayed antagonistic activity (as judged by inhibition of growth) against three or more marine bacterial isolates derived from sponges (Table 3.7). This suggests that in the sponge microbial community itself, the compounds responsible for this activity may also be produced and may result in antagonism. Such activity may be significant in controlling the structure of the microbial community within the mesohyl of the sponge (Jensen and Fenical, 1994; Engel *et al.*, 2002).

Bacteria employ antagonistic interactions against other bacteria, perhaps to limit competition in the nutrient-rich microenvironments (Patterson and Bolis, 1997). Several studies have demonstrated that certain strains of bacteria can be induced to produce antibiotics (Mearns-Spragg *et al.*, 1997; Mearns-Spragg *et al.*, 1998). These were due to response to the chemical signals received from potential competitor strains, which elicit an antagonistic response. Previous studies indicated that chemical interactions between different species of bacteria can affect the production and secretion of antimicrobial secondary metabolites (Patterson and Bolis, 1997; Mearns-Spragg *et al.*, 1998; Burgess *et al.*, 1999). Other work considered that the enhancement of antimicrobial compound production by bacteria, when they are exposed to a different strain of bacteria, suggested that competition for space between epibiotic bacteria might provide further antifouling protection to the basibiont (Armstrong *et al.*, 2001).

It has been noticed that some bacteria that previously did not produce any active compounds have been found to produce such metabolites when they are exposed to other bacterial species or extracellular products from other bacteria (Mearns-Spragg *et al.*, 1998;

Armstrong *et al.*, 2001; Yan *et al.*, 2002; Xue *et al.*, 2003). They concluded that the exposure of seaweed-associated bacteria on surface of seaweed increased the number of screening hits of antimicrobial compound producers. Results from this study demonstrated that strains *B. licheniformis*, SC-43 and *B. subtilis*, SD-8 could release antimicrobial compounds when grown in sessile biofilms, which might help the bacterial community establishment in the sponges.

In this study, results indicated that all the strains tested were active against the indicator strain *M. luteus*, which was also isolated from sponge. Other studies demonstrated the same activity from two of the sponge species, *Axinella corrugate* and *H.* aff *tubifera* against *M. luteus* (Monks *et al.*, 2002). This is consistent with other studies, suggesting that *M. luteus* can be routinely used in the laboratory as a sensitive indicator of activity (Ross *et al.*, 1993; Navaratna *et al.*, 1998).

4.2.2 Production of antimicrobial compounds from bacterial isolates using various techniques

NGF was the best medium for isolating bacteria producing antimicrobial and for most sponge bacterial isolates in this study. These suggest that glycerol and ferric ion were very important sources for the production of bioactive compounds. The same phenomenon was reported that glycerol and ferric ion were essential for a *Bacillus licheniformis* strain EI-34-6, isolated from the surface of the red seaweed *Palmaria palmata*, to produce bacitracin when grown within a biofilm (Yan *et al.*, 2003). Their findings of the dose response effect of ferric ion and glycerol on the production of bioactive compounds confirmed their involvement in the synthesis of bacitracin.

There are few studies showing why ferric ion and glycerol are essential for the production of antimicrobial compounds. In my study there were three strains, *B. licheniformis*, SC-43, *B. subtilis*, SD-8 and *Pantoea* sp., SC-AF which needed both glycerol and ferric ions to produce antimicrobial compounds. Further studies are necessary to investigate the mechanisms of glycerol and ferric ion in eliciting the production of antimicrobial compounds by marine bacterial isolates.

Previous studies have indicated that the production of secondary metabolites and spore formation are related in bacilli (Msadek, 1999; Demain and Fang, 2000; Yan *et al.*, 2003). It has been found that release of secondary metabolites by bacilli and sporulation are regulated by similar factors. This similarity could ensure secondary metabolite production during sporulation. Thus, some secondary metabolites may be involved in suppression of germination until a less competitive environment and more favourable conditions for growth exist. However, other findings also suggest that production of certain antibiotic compounds by bacilli may not depend on sporulation (Yan *et al.*, 2002; Yan *et al.*, 2003). These authors suggest that regardless of sporulation level, the supernatant did not exhibit antimicrobial activity when a *B. licheniformis* strain EI-34-6 was grown under the planktonic suspension state. In contrast with AMS culture, at any percentage of sporulation level, the production of antimicrobial compounds by *B. licheniformis* strain EI-34-6 was not affected. In this study the same pattern was observed, which suggests that under certain condition sporulation may not play a role in the production of antimicrobial compounds. Previous studies show that the use of seawater in the production medium enhanced the production of the activity of the halotolerant strain of *Bacillus licheniformis* by 150% (Manachini and Fortina, 1998).

In total, my results are not in agreement with the previous results described above. In this study *B. licheniformis* SC-43, *B subtilis* SD-8 and *Pantoea* sp., SC-AF did not exhibit antimicrobial compounds after the use of seawater in the media. In fact, several media namely Marine broth, Nutrient broth, SYZ broth, NGF broth, M-A1 broth, M-A2 broth, Artificial seawater (ASW) broth and Natural seawater (NSW) broth were chosen for production of antimicrobial compounds and were prepared using both natural and artificial seawater. However, the three strains did not show significant differences in the production of antimicrobial compounds when cultured using either of the seawater based media. Only two media, M-A2 and NGF gave a significant result in terms of antimicrobial activity. However, the activity obtained using M-A2 was not as high as that using NGF medium. Therefore, elicitation of antimicrobial compounds by the three strains did not depend on salinity, but was affected by glycerol and ferric ion.

Previous findings have also indicated that Gram-positive bacteria isolated from the sea do not require seawater or sodium for growth (Marquez *et al.*, 1990). Earlier work has shown that the addition of supplements such as sorbitol, sodium chloride, methionine and manganese enhanced the production of certain enzymes such as *L*-glutaminase and chitinase (Keerthi *et al.*, 1999; César and Facundo, 2003). The problem lies in how to provide bioactive compound producers with the proper environmental conditions. Some studies suggested that 'niche-mimic' bioreactors, which are designed to mimic ecological niches of bacteria, may provide the opportunity to elicit production of antimicrobial compounds by some marine bacterial isolates (Yan *et al.*, 2003).

Bioprocess engineering in marine biotechnology has great potential in the design and optimization of the bioreactors for marine metabolite production. A variety of bioreactor designs have been implemented with varying degrees of success. The opportunity to produce new bioactive structural analogues of known compounds via manipulation of culture conditions presents marine biotechnologists with a unique challenge for new bioproduct discovery. Innovations in media development, bioreactor design, and transgenic production, coupled with efficient downstream processing and product recovery, will be necessary to meet the needs of both discovery and bulk production of novel marine bioproducts (Pomponi, 1998). By using the AMS bioreactor, results indicated that three species of bacteria, *B. subtilis* SD-8, *B licheniformis,* SC-43 and *Pantoea* sp., SC-AF produced antimicrobial compounds in NGF medium (Table 3.8). Several other media were also used to grow the sponge bacterial isolates to test production of antimicrobial compounds. However, they did not release any detectable antimicrobial compounds when grown using Marine broth, SYZ broth, Nutrient broth, ASW broth, and NSW broth, suggesting that glycerol and ferric ion were needed for the production of bioactive compounds. This is in agreement with a previous report (Yan *et al.*, 2003).

It has been found that *B. subtilis* SD-8, *B licheniformis,* SC-43 and *Pantoea* sp., SC-AF only produce antimicrobial compounds when grown in aerobic conditions. I deduced that the link with ferric ion, glycerol and oxygen could be the oxidative stress, and production of antimicrobial compounds by these three strains might be "oxidative stress eliciting". Oxidative stress is defined as an excess of prooxidants in the cell such as superoxide anion

(O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Storz and Imlay, 1999). These reactive oxygen intermediates have a high oxidizing potential and thus are responsible for cellular oxygen toxicity (Farr and Kogoma, 1991).

On the other hand, bacterial stress can be defined as physiological perturbation caused by environmental modifications (physical, chemical and/or nutritional) that can have many consequences for the bacteria, such as retarded growth and cell death (Farr and Kogoma, 1991; Fridovich, 1998; Storz and Imlay, 1999). To counter oxidative stress, cell constitutively express enzymes that detoxify the reactive oxygen species and repair the damage caused by them.

In this study glycerol could be oxidised to a superoxide generator, glyceraldehyde. This short chain sugar and its phosphate esters, in which the carbonyl group is not largely blocked by cyclization to the furanose or to more stable pyranose rings, can tautomerize to enediols, which are prone to autoxidation. O_2^- has been shown to serve as both an initiator and the propagator of these autoxidations (Mashino and Fridovich, 1987). Short chain sugars are thus potentially capable of synergizing with O_2^- in causing toxicity. O_2^- acts as a nucleophile as either oxidizing or a reducing agent. In aqueous environments (broth), O_2^- reacts with itself to produce O_2 and H_2O_2. The subsequent reaction of H_2O_2 with Fe^{2+} as transition metal, results in the production of the hydroxyl radical (OH^-), a powerful oxidant that reacts with most molecules. This is dangerous because they can initiate an autocatalytic radical chain reaction and will damage the cell.

To protect against the damage caused by oxidative stress, cells possess a number of antioxidant enzymes and repair activities, most of which are expressed at low levels during normal growth. In response to elevated concentrations of O_2^- and H_2O_2, the expression of many antioxidant proteins is induced. Among O_2^- inducible activities are manganese superoxide dismutase (*sod*A) and iron superoxide dismutase (*sod*B). It is clear that one of the functions of the SODs is to protect the metabolic intermediates against oxidations initiated and or propagated by O_2^- (Benov and Fridovich, 1998) and reduce the level of O_2^- In this study the oxidization of glyceraldehyde and the formation of O_2^- lead to environment stress. Therefore, the production of antimicrobial compounds may suggest that

oxidative stress can elicit the production of different secondary metabolites. These secondary metabolites, although some of them can show antimicrobial activity, are originally by-products during anti-oxidative metabolism, rather than killing their competitors.

Previous study indicated that most bacteria exist attached to surfaces within biofilms and are inherently different from bacteria existing in the planktonic state (Korber *et al.*, 1995). In line with that, some studies also indicated that the addition of sand to shake flask cultures induced exopolymer synthesis by some surface-isolated bacteria and that exopolymer production by attached cells was greater than that by the same bacteria growing in the planktonic state (Vandevivere and Kirchman, 1993). Other studies also tried to grow bacteria in a sessile condition by using modified roller bottle cultivation. Consequently, bacteria produced bioactive compounds under surface attached conditions (Yan *et al.*, 2002). These authors introduced the conditions by which periodic exposure to liquid medium and to air clearly led to the production of bioactive compounds. This type of reactor is known as `niche mimic bioreactor`. I have tried different kind of substrate by using loosely artificial sponges as well as the nylon membrane and submerged into the medium of shake flask culture. However, this study demonstrated that by putting loosely artificial sponge or nylon membrane into the shake flask culture did not exhibit antimicrobial compounds. This suggests that some of the bacteria involved in the production of antimicrobial compounds did not rely on solid substrate to induce synthesis of compounds. Therefore, other critical reasons for antimicrobial compound production by these isolates should be present.

In this study the supplements responsible for the production of the inhibitory compounds on marine agar by three species, *B. licheniformis*, SC-AF, *B. subtilis*, SD-8 and *Pantoea* sp., SC-43, were shown to be glycerol and ferric ion. They seemed to be synergetic. It was indeed demonstrated that glycerol alone would not enhanced the bioactive compounds. Apparently, no other factor was involved, since it was also found that pre-supplementation of nutrient broth of SC-AF, SD-8 and SC-43 with various concentrations of glycerol or ferric ion alone did not lead to enhanced bioactive compounds production.

Other work has shown that when *B. licheniformis*, strain EI-34-6, isolated from the surface of *Palmaria palmata*, was grown in an AMS bioreactor, the cells produced bacitracin, which they did not produce when they were grown in shake flask cultures. However, the cell-free supernatant from the AMS culture (including glycerol and ferric metabolites) could elicit the production of bacitracin in corresponding shake flask cultures (Yan *et al.*, 2003). The same phenomenon was observed in my study. Further study at Heriot-Watt University (Sara, M. pers. comm.) has shown menadione, paraquat and glyceraldehyde were able to replace cell-free supernatant from the AMS culture and mimic the bacitracin elicitation by EI-34-6 in shake flask cultures. Menadione, paraquat and glyceraldehyde possess very different chemical structures and go though very different metabolic pathways. However, they share one common property that they all can complete autoredoxing and can be superoxide generators when supplemented in aerobic bacterial cultures.

One explanation to unrevealing the complex response to produce different secondary metabolites by a biofilm in AMS bioreactor is the involvement of autoredoxing compounds. It might take place of O_2 as electron recipient than partially help the oxidation of lower layer of biofilm. In addition, bacterial cells grown using AMS culture established a compulsory biofilm in contact with the air. Therefore the bacterial cells layer in contact with the air on the top will have a sufficient oxygen supply. However, oxygen might not diffuse easily into the biofilm, resulting in inadequate oxygen provision at the bottom layer of the biofilm. Therefore, an oxygen gradient will be established.

On the contrary, bacteria grown at the bottom layer of the biofilm can have enough nutrient supply, but due to the diffusion difficulty of the nutrient from the bottom layer to the top layer of the biofilm, the cells grown within the top layer are comparatively short of nutrient supply. Therefore, the cells grown within the biofilm will subjected to a nutrient gradient opposite to that of the oxygen gradient. Therefore, the metabolic pattern of the cells within the biofilm will be significantly different between each layer. These types of physical conditions for the growth of the biofilm may result in the metabolic difference from that using planktonic suspension culture.

The bioreactor design based on the biofilm establishment may make more opportunity to produce novel secondary metabolites or increase the yield of some compounds currently being produced using the agitated planktonic suspension cultivation method. Three of the bacterial isolates, *B. subtilis* SD-8, *B licheniformis* SC-43 and *Pantoae* sp., SC-AF did not produce any antimicrobial compounds when grown in the shake flask cultures but they did in the ReacSyn®-Bioreactor. This was supported by several studies indicating that bioreactor designs affect the production of bioactive compounds (Pomponi, 1998; Yan *et al.*, 2003).

The bioreactor design that allows bacteria to grow attached to a surface as a biofilm in contact with air thus mimicking the intertidal environment, could facilitate the production of antimicrobial compounds (Yan *et al.*, 2002). Thus, the design of a bioreactor is to give novel physical conditions that allow production of secondary metabolites. Furthermore, the unique growth conditions when cultivated cells are attached directly or indirectly to the solid medium and those cells can form a biofilm is an important factor in the production of antimicrobial compounds as indicated by two strains in this study. Culture supernatant from AMS culture of strains *B. subtilis*, SD-8 and *B. licheniformis*, SC-43 exhibited an antibiotic spectrum different from the correspondent cultures using the ReacSyn®-Bioreactor. The present study indicated that both bacteria, SD-8 and SC-43 were producing inhibitory compounds against the multi-drug resistant bacteria, MRSA but not against VRE (Table 3.7, Figure 3.14).

Studies have been conducted to compare secondary metabolite production by *Bacillus* sp. in planktonic suspension cultures and sessile biofilm cultures (Armstrong *et al.*, 2001; Yan *et al.*, 2002; Yan *et al.*, 2003). It is clear that changes in the mode of cultivation can affect the nature and amount of compounds that bacteria produce (Burgess *et al.*, 1999). It has been further demonstrated that bacteria grown on surfaces released bioactive compounds that have greater activity against target strains compared with those from the same strain grown in the shake flask cultures. Thus, these results are in agreement with my finding that the AMS culture supernatant exhibited antimicrobial compounds but not in shake flask culture supernatant.

This may be due to the different gene expression which might be involved in extracellular polysaccharide production once the bacteria have settled on the surface or resided in the host. In this study under growth conditions of planktonic suspension, ReacSyn®-Bioreactor and AMS bioreactor, three bacterial strains, *B. licheniformis* SC-43, *B. subtilis* SD-8 and *Pantoea* sp., SC-AF, apparently produced different secondary metabolites. In addition, only AMS culture maintained the production of bioactive compounds under the same physiochemical parameters such as carbon and nitrogen sources, temperature and oxygen supply. However, planktonic suspension (shake flask culture) did not facilitate production of any antimicrobial compounds.

In this study a semi-permeable membrane was chosen for the ReacSyn®-Bioreactor to get the best production of antimicrobial compounds. Results indicated that the nylon membrane was the best membrane (as AMS Bioreactor membrane) and exhibited the production of bioactive compounds suggested that components of membrane gave affect on the production of bioactive compounds. Although ReacSyn®-Bioreactor gave similar results as AMS culture, however due to the design, sterility has been difficult to maintain. Thus, the culture was difficult to scale-up. It has been postulated that oxidative stress might facilitate bacteria to produce bioactive compounds upon attachment to the substrate.

Chemical interaction was also shown between bacterial species. The interaction could elicit production of various secondary metabolites by bacteria, including antimicrobial compounds. It has been first demonstrated in *Vibrio harveyi* which were induced luminescence by culture supernatants of non-luminescent *Vibrio* sp. (Bassler *et al.*, 1997). It has been noticed that exposure of marine bacteria to terrestrial bacteria can produce cross-species induction and enhancement of the production of secondary metabolites (Billaud and Austin, 1990). This indicates that bacteria are able to express and produce antimicrobial compounds with the help of other species of the same genus (Mearns-Spragg, 2000). A few studies have been conducted to prove these phenomena (Mearns-Spragg *et al.*, 1998; Yan *et al.*, 2003).

Cell-cell communication between the same species of bacteria to stimulate a number of physiological and biochemical activities in bacterial populations is well understood

(Kaprelyants and Kell, 1996; Miller and Bassler, 2001; Whitehead *et al.*, 2001, Taylor *et al.*, 2004a). In this study an attempt was made to use cell-free supernatant produced by *B. licheniformis,* SC-43, *B. subtilis,* SD-8 and *Pantoea* sp., SC-AF in AMS cultures to elicit antibiotic production in the correspondent shake flask culture. It has been noticed from the observations that only *B. licheniformis,* SC-43 and *B. subtilis,* SD-8 exhibited antimicrobial compounds from those conditions. In addition to this observation, *Pantoea* sp., SC-AF seemed to enhance the production of extracellular polysaccharide and the shake flask culture was getting significantly viscous. This indicated that the production of polysaccharide increased suggesting the involvement of microbial antagonism between them and inhibits the production of any useful compounds. However, it is important to note that ferric ion and glycerol play major roles in the production of antimicrobial compounds for two strains of *Bacillus*. Furthermore in this study, glycerol or ferric ion alone in the media did not exhibit any antimicrobial compounds, which suggested that both formation of inducing levels of bioactive compounds must occur in liquid cultures in conjunction with ferric ion and glycerol. In fact this has been reported by a previous study (Yan *et al.*, 2003).

It also suggested the production of autoinducers should be the key requirement that allows EI-34-6 grown in the AMS bioreactor to produce antimicrobial compounds and the red pigment. From this point of view, it seems that at least three factor are involved in the production of antimicrobial compounds which are ferric ion, glycerol and the growth in a biofilm formed at the air-membrane interface. The use of different kinds of media failed to promote the production of useful compound suggesting that glycerol and ferric ion were the only factors inducing the production of antimicrobial compounds. From the use of different kinds of cultivation methods, it has been shown that formation of a biofilm at the air-membrane surface has led to production of antimicrobial compounds. Unfortunately, the degree to which antimicrobial activities translate to *in situ* effects upon potential surface-colonizing bacteria has not been adequately demonstrated.

4.3 STUDY OF THE PRODUCTION OF ANTIMICROBIAL COMPOUNDS BY *Pantoea* sp., SC-AF ISOLATED FROM THE SPONGE, *S. carnosus*

4.3.1 Characterization of Pantoea sp., SC-AF

The phenotypic characteristics of SC-AF are typical of the Enterobacteriaceae as described (Hauben *et al.*, 1998). Based on the 16S rDNA sequence, SC-AF falls in a phylogenetic cluster (above 95% sequence identity) comprising *Pantoea* spp. including *P. agglomerans*, *P. ananatis* and *P. dispersa*. 16S rDNA sequencing analysis of SC-AF showed 99.5 % similarity to a strain designated as *P. ananatis* (Accession Number: AY530798). However, SC-AF differs from *P. ananatis* in defining characteristics, including oxidase negative, production of polysaccharide, forms colonies that are strongly attached to the agar surface, and catabolism of glucose with no gas production (Hauben *et al.*, 1998). It is concluded that this SC-AF strain can produced extraordinary amount of extracellular polysaccharides (EPS) compared with other isolates. This seems the first report of a *Pantoea* sp. isolated from sponges.

In this study results indicated that NGF and medium A-2 were the only media that could elicit the production of antimicrobial compounds. Compared with NGF, SC-AF produced very weak antimicrobial compounds in medium A-2. This suggests that the antimicrobial compounds produced by SC-AF were inducible by the chemical environment surrounding the bacteria. Several experiments were also conducted to observe the affect of metal ion in terms of the production of antimicrobial compounds. Manganese, Sodium nitrate, and Ferric ion were added to the Nutrient Broth and Glycerol media. Results indicated that only ferric ion gave a significant affect suggesting the involvement of this ion in the production of antimicrobial compounds. The result also suggests that SC-AF produced antimicrobial compounds when grown using the AMS culture method. It suggested that surface attachment, exposure to the air and biofilm formation may play an important role in eliciting the production of antimicrobial compounds. This result also suggests that SC-AF might produce antimicrobial compounds in its natural niche grown in *S. carnosus* when attached to a surface in a biofilm, but might not when living in 'free-swimming condition'.

The AMS bioreactor, which incorporated some of the natural niche of *Pantoea* sp., SC-AF provides a membrane surface upon which to grow. In the natural niche environment, *Pantoea* sp., SC-AF should have a sessile living condition attached to the mesohyl, which will lead to formation of a biofilm. From this aspect, the AMS bioreactor partially mimics the natural niche. The growth of SC-AF within a biofilm and production of EPS seems to be related. It has been observed that with the increase of the production of EPS the production of antimicrobial compounds reduced. It is suggested that both were inversely correlated. An antagonistic relationship between the production of antimicrobial compounds and production of EPS could lead to the survival of this bacterium in sponge-bacteria communities.

Another study has suggested that production of EPS can protect bacteria from various stresses (Amellal *et al.*, 1998). Perhaps, the low concentrations of antimicrobial compounds in the supernatants could be explained by the increased production of EPS by *Pantoea* sp., SC-AF. A previous study indicated that a *Pseudomonas* sp. strain increased its polysaccharide production during desiccation (Roberson and Firestone, 1992). The production of EPS possibly enhances water retention in the microbial environment and seems to regulate the diffusion of a carbon source such as glucose (Chenu and Roberson, 1996). On the other hand, it was shown previously that microbial biomass and polysaccharide production increased in associations of microbial populations in the rhizosphere of various plants (Haynes and Swift, 1990). EPS improve the nutrient supply to cells during desiccation events and trap dissolved nutrients as they hold large amounts of water at low water potentials. They are able to bind cations (Ca^{2+} and Mg^{2+}) to their negatively-charged sites. EPS also increase the outer surface area of the cell and the numbers of contact points with the soil matrix (Chenu and Roberson, 1996). All this may help bacteria to maintain physiological function and partially compensate for the substrate limitation that occurs.

4.3.1 Characterization of antimicrobial compounds of Pantoea sp., SC-AF

Fractionation using molecular size filtration (Amicon Ultra-15 (Millipore) showed active compounds were present in cell free supernatant (CFS) with fraction sizes of >5 kDa - < 5 kDa. Both fractions gave a significant inhibition zone on disc diffusion test. It suggested that there were at least two antibiotic compounds of different sizes produced by SC-AF in AMS cultures. It showed that more active antimicrobial compounds were filtered into fractions less than 5 kDa, which suggests low molecular weight compounds. However it was observed during filtration of the Amicon Ultra-15 (Millipore) with a cut-off of 5 kDa, that water was still left in the fraction in which the compounds with molecular weight > 5 kDa should be present. This suggested this filtration system is aiming at concentrating big molecules (in this example, > 5 kDa). However, it did not necessarily separate small molecules with molecular size < 5 kDa completely simply because molecular weight of water is only 18, far less than 5 kDa. The accumulation of water in the fraction > 5kDa might be because the big molecules (> 5 kDa) blocked the pores in the filtration system. In addition, the fraction of small molecules (< 5 kDa) was active. Therefore, there was at least one small active molecule present in the supernatant. However, the possibility that this small molecule was not filtered through still existed. Thus, although the fraction containing big molecules (> 5 kDa) was active, it is hard to draw the conclusion that this activity was due to large molecules (> 5 kDa). Because of this uncertainty Amicon Ultra-15 is not a reliable method to fractionate small molecule.

The earliest studies on other antibiotics produced by strains of *Pantoea agglomerans* show that they are also likely to be low molecular weight compounds with a peptide component (Wright *et al.*, 2001). Similar reports suggest the majority of compounds are peptides of low molecular weight from different group of bacteria which are either ribosomally synthesized such as the lantibiotics, which contain a modified amino acid lanthionine residue, or are non-ribosomally synthesized, employing large enzyme complexes, such as vancomycin (Jack and Jung, 2000; Mannanov and Sattarova, 2001; Salomon *et al.*, 2004). Thus, in this study focus was on initial purification of antimicrobial peptide-containing compounds. Another reports suggest that some strains of *P. agglomerans* and *Pantoea* spp. produce single antibiotics such as strain Eh252 whereas others produce multiple

compounds inhibitory against *E. amylovora* (Vanneste *et al.*, 1992; Kearns and Hale, 1996). *P. agglomerans* strain Eh318 produces two antibiotics that are active against *Erwinia amylora* Ea273, namely Pantocin A and Pantocin B. The structure of Pantocin A is unknown, whereas that of Pantocin B has been determined as R-N- ((S)-2-amino-propanoylamino)-methyl)- 2-methanesulfonyl-succinamid acid. It was recently identified as a peptide of 296 Da (Brady *et al.*, 1999). Antibiotics of *Pantoea* species are frequently grouped on the basis of the type of amino acid involved. As an example, most strains of *P. agglomerans* and *P. dispersa* produce histidine-reversible or histidine and/or leucine-reversible antibiotics (Wodzinski and Paulin, 1994). Histidine reverses the activity of Pantocin A and arginine reverses that of Pantocin B.

Antibiotic activity of CFS from *Pantoea* sp., SC-AF, *B. licheniformis* SC-43 and *B. subtilis* SD-8 was retained after being heated at 100°C for five minutes and autoclaved at 121°C. It is suggested that all these antimicrobial compounds produced by the three strains cannot be protein. This has been shown on some of the peptides such as subtilin from *B. subtilis* (Nakano and Zuber, 1990). In contrast, other study reported that some strains of *P. agglomerans* are sensitive to the heat (Kearns and Hale, 1996). The active compounds produced by SC-AF were not able to be extracted from the culture supernatant using ethyl acetate or butanol, which suggested that the compounds were very polar. Further C18 Sep-Pak® fractionation also showed the active fraction was eluted off the cartridge only at 30% CH_3CN: 70% H_2O gradient, which suggests that the compound polarity was very high.

In this study six peaks present in the analytical HPLC exhibited antimicrobial activity, which suggested that the activity was due to six different compounds. However, so far only *P. agglomerans* strain C9-1 produced three different antibiotics which were purified and characterized preliminarily (Ishimaru *et al.*, 1988). Previous study indicated that antibiotics of several *Pantoea* strains were initially designated bacteriocins due to their inhibition primarily of closely related species (Beer and Rundle, 1980). However, different strains of *Pantoea* might produce different type of antimicrobial compounds. This was clearly observed from two species of *Pantoea*: *P. agglomerans* and *P. dispersa.* They were reported to produce different spectra of antimicrobial compounds which gave different types of inhibition zones against the same indicator organisms (Beer and Rundle, 1980; El-

Goorani *et al.*, 1992). In my study the antimicrobial compounds produced by SC-AF showed significant activity against many Gram-positive bacteria rather than closely related species. In addition, the heat resistant property also suggested non-protein components. Therefore, the antimicrobial I have been trying to characterise is very unlikely to be bacteriocin.

High pressure was applied in HPLC to give better separation of samples. In this study the results obtained were not pure enough; suggesting HPLC is not good enough for purification. Thus, other mechanisms must be used for preliminary purification such as silica gel. Silica gel based on hydroxyl and polarity might improve the performance of HPLC.

4.4 POLYSACCHARIDE PRODUCTION BY *Pantoea* sp., SC-AF

In this study two types of sugar, fructose and cellobiose, were characterized in their monosaccharide component. No other sugar was detected from the HPLC result suggesting a limitation of the sugar profile through using HPLC. Another study suggests that sugar, including monosaccharides present in low proportions, are generally not quantified accurately as this is difficult to accomplish except by the HPLC-Electrochemical detection method (HPLC-ED) (Bazzanella and Bachmann, 1998). However, a further study indicated that EPS stewartan from *P. stewartii* subsp. *stewartii* consists of a seven monosaccharide repeating unit that contains glucose, galactose, and glucuronic acid (Coplin and Majerczak, 2002). This suggests that different EPS from different species might have different types of sugar.

4.5 PRELIMINARY STUDY OF PROTON (^{1}H) NMR AND MS

There were more than six continuous peaks after HPLC fractionation showed activity. Therefore, at least six active components were present in the culture. They possibly belong to the same chemical family and share a similar structure bone. The yield of this antimicrobial compound by SC-AF was not very high. From a two litre AMS culture there were only about 1~1.2 mg pure compound obtained. It can be deduced that in order to obtain enough amount of the antimicrobial (8 mg), 15~16 litre AMS culture will be necessary. The current AMS cultures are carried out using 8ml dishes. Therefore, 2,000 dishes have to be inoculated. Thus it will be very important to find out inducers to elicit this antimicrobial production in shake flask culture, or to carry out screening work to select mutants with high production yield.

Two active fractions separated from HPLC did not contain components with molecular weight of 296 (Pantocin B). In addition, base peaks of Fr-1 and Fr-2 are 385.4 and 423.4 respectively which could not be any fragments resulting from Pantocin B. Therefore MS also confirmed proton (^{1}H) NMR result that antimicrobial compounds produced by *Pantoea* sp., SC-AF was not Pantocin B.

CHAPTER 5 CONCLUSIONS

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1. Amongst several media tested, Marine Agar was the best medium for cultivation of culturable bacterial isolates from the sponges but Nutrient-Glycerol-Ferric broth (NGF) was found to allow more isolates to produce antimicrobial compounds.

2. Both culture-dependent and culture-independent strategies are indispensable for study of the ecology of the microbial community of sponge associated bacteria.

3. *Bacillus* spp. (40%) were the dominant cultured Gram-positive bacteria from the four studied sponges, *H. panicea*, *S. domuncula*, *S. carnosus* and *P. johntonia*.

4. Most of the uncultured bacteria were affiliated with *Synechococcus* sp., a group of cyanobacteria.

5. 90% of the bacteria associated with the sponges *H. panicea*, *S. domuncula*, *S. carnosus* and *P. johntonia* were species-specific.

6. The denaturing gradient gel electrophoresis (DGGE) method provided good characterization of the assemblage and diversity of marine sponge-associated bacteria, for comparative studies but was not found to be good for identification of bacteria.

7. Bacterial communities of *S. carnosus* changed gradually after being transferred to an aquarium. To change the surrounding environment of the sponge can be a good strategies to reveal potential bacterial symbionts.

8. A *Micrococcus luteus* strain, HP-5/6, isolated from *H. panicea* can be used in the laboratory as a sensitive indicator of activity.

9. Ferric ion, glycerol and being grown in a biofilm at the air-solid interface were found to be three essentials factors involved in the production of antimicrobial compounds by *B. licheniformis* (SC-43), *B. subtilis* (SD-8), and *Pantoea* sp., SC-AF.

10. *Pantoea* sp., SC-AF produced antibiotics along with the production of extracellular polysaccharides (EPS) when grown within a biofilm at the air-membrane surface.

11. Only fructose and cellobiose have been identified after acid lysis of EPS of *Pantoea* sp., SC-AF.

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APPENDICES

Appendix 1: Strain identification, (*Halichondria panicea* (HP), *H. bowerbanki* (HB), *Suberites domuncula* (SD), *S. carnosus* (SC), *Dysidea avara* (DA), *Chondrosia reniformis* (CR), *Axinella polypoides* (AP), *Clione celata* (CC), *Pachymatisma johnstoni* (PJ)), morphologies, Gram status, source of the isolates and medium for isolation are listed.

Isolate ID	Colony colour	Morphology	Elevation	Gram-stain	Isolation medium	Source of isolate
HP-1	White	Round	Raised	Positive	MA	HP
HP-2	Beige	Round	Raised	Positive	MA	HP
HP-3	Beige	Oval	Convex	Positive	MA	HP
HP-4	Yellowish	Oval	Raised	Positive	MA	HP
HP-5	White	Irregular	Flat	Positive	MA	HP
HP-6	Orange	Round	Raised	Positive	MA	HP
HP-7	Beige	Round	Raised	Positive	NA	HP
HP-8	Beige	Oval	Raised	Positive	NA	HP
HP-9	Brown	Round	Raised	Positive	SWA	HP
HP-10	Brown	Round	Raised	Positive	SWA	HP
HP-11	Greenish	Round	Flat	Positive	NA	HP
HP-13	White	Round	Flat	Positive	NA	HP
HP-14	Beige	Round	Raised	Positive	YPSS	HP
HP-15	Beige	Round	Raised	Positive	YPSS	HP
HP-17	Yellow	Irregular	Raised	Positive	YPSS	HP
HP-18	White	Round	Raised	Positive	YME	HP
HP-19	Yellow	Round	Raised	Positive	MA	HP
HP-20	Beige	Irregular	Raised	Positive	YME	HP
HP-22	Beige	Oval	Raised	Positive	MA	HP
HP-23	Beige	Oval	Raised	Positive	TSW	HP
HP-24	Beige	Round	Raised	Positive	TSW	HP
HP-25	Beige	Round	Raised	Positive	YME	HP
HP-27	Beige	Round	Raised	Positive	NA	HP
HP-28	Yellow	Irregular	Raised	Positive	MA	HP
HP-29	Colourless	Oval	Raised	Positive	NA	HP
HP-30	Beige	Oval	Raised	Positive	NA	HP

Isolate ID	Colony colour	Morphology	Elevation	Gram-stain	Isolation medium	Source of isolate
HP-31	White	Irregular	Flat	Positive	NGF	HP
HP-43	Orange	Round	Flat	Positive	SWA	HP
HP-49	Beige	Oval	Raised	Positive	NA	HP
HP-51	Brown	Oval	Raised	Positive	NGF	HP
HP-53	Red	Irregular	Raised	Positive	NGF	HP
HP-67	Red	Irregular	Raised	Positive	NGF	HP
HP-72	Yellow	Irregular	Raised	Positive	MA	HP
HP-73	Beige	Oval	Raised	Positive	YPSS	HP
HP-89	Beige	Round	Flat	Positive	SWA	HP
HP-99	Beige	Round	Raised	Positive	NA	HP
HP-112	White	Round	Raised	Positive	TSW	HP
HP-137	White	Round	Raised	Positive	MA	HP
HP-153	Beige	Round	Raised	Positive	NA	HP
HP-180	Beige	Round	Raised	Positive	NGF	HP
HP-200	Beige	Irregular	Raised	Positive	NGF	HP
HP-207	Yellowish	Round	Raised	Positive	NGF	HP
HP-208	Colourless	Round	Raised	Positive	NGF	HP
HP-215	Beige	Round	Flat	Positive	MA	HP
HP-220	White	Oval	Flat	Positive	SWA	HP
HP-221	White	Round	Flat	Positive	YPSS	HP
HP-226	Beige	Round	Flat	Positive	NA	HP
HP-229	Beige	Round	Raised	Positive	YME	HP
HP-230	Beige	Round	Raised	Positive	MA	HP
HP-233	Yellow	Irregular	Raised	Positive	MA	HP
HP-5/6	Yellow	Irregular	Raised	Positive	MA	HP
HB-5	White	Round	Raised	Positive	NA	HB
HB-12	White	Round	Raised	Positive	NA	HB
HB-19	White	Round	Raised	Positive	NA	HB
HB-23	Colourless	Oval	Flat	Positive	MA	HB
HB-27	Colourless	Round	Flat	Positive	MA	HB

Isolate ID	Colony colour	Morphology	Elevation	Gram-stain	Isolation medium	Source of isolate
HB-32	Beige	Round	Raised	Positive	MA	HB
HB-55	Orange	Irregular	Raised	Positive	SWA	HB
HB-57	Yellow	Oval	Flat	Positive	NGF	HB
HB-70	Beige	Round	Flat	Positive	NGF	HB
HB-72	Beige	Round	Flat	Positive	MA	HB
HB-78	Beige	Round	Raised	Positive	MA	HB
HB-87	Beige	Oval	Raised	Positive	SWA	HB
HB-90	Beige	Round	Raised	Positive	MA	HB
SD-1	White	Oval	Raised	Positive	MA	SD
SD-2	Orange	Round	Flat	Positive	MA	SD
SD-5	Colourless	Round	Flat	Positive	NA	SD
SD-6	White	Round	Flat	Positive	MA	SD
SD-7	Orange	Oval	Raised	Positive	NA	SD
SD-8	Brown	Round	Raised	Positive	NA	SD
SD-9	White	Round	Raised	Positive	NA	SD
SD-10	White	Round	Raised	Positive	SWA	SD
SD-11	Yellow	Irregular	Raised	Positive	MA	SD
SD-12	Beige	Round	Raised	Positive	NGF	SD
SD-18	White	Oval	Raised	Positive	NGF	SD
SD-19	Colourless	Irregular	Raised	Positive	NGF	SD
SD-20	Beige	Round	Raised	Positive	YPSS	SD
SD-22	Beige	Round	Raised	Positive	YPSS	SD
SD-24	Beige	Round	Raised	Positive	YPSS	SD
SD-26	Pink	Oval	Raised	Positive	SWA	SD
SD-27	White	Round	Raised	Positive	YME	SD
SD-28	White	Irregular	Raised	Positive	TSW	SD
SD-29	Beige	Oval	Raised	Positive	SWA	SD
SD-32	Colourless	Round	Convex	Positive	MA	SD
SD-35	Greenish	Round	Raised	Positive	MA	SD
SD-39	Beige	Round	Flat	Positive	MA	SD

Isolate ID	Colony colour	Morphology	Elevation	Gram-stain	Isolation medium	Source of isolate
SD-45	Beige	Round	Raised	Positive	SWA	SD
SD-57	Beige	Round	Raised	Positive	MA	SD
SD-58	White	Round	Raised	Positive	MA	SD
SD-62	White	Oval	Convex	Positive	NA	SD
SD-65	White	Oval	Convex	Positive	NA	SD
SD-69	Greenish	Round	Raised	Positive	YME	SD
SD-78	White	Oval	Flat	Positive	MA	SD
SD-84	White	Oval	Flat	Positive	MA	SD
SD-85	Yellowish	Round	Raised	Positive	YPSS	SD
SD-89	Yellowish	Round	Raised	Positive	YPSS	SD
SD-113	Beige	Oval	Flat	Positive	TSA	SD
SD-121	White	Irregular	Raised	Positive	MA	SD
SD-140	Beige	Oval	Flat	Positive	TSW	SD
SD-142	Beige	Oval	Flat	Positive	NA	SD
SD-181	White	Irregular	Raised	Positive	MA	SD
SD-193	White	Irregular	Raised	Positive	MA	SD
SD-200	White	Round	Flat	Positive	MA	SD
SD-212	Yellow	Round	Flat	Positive	NGF	SD
SD-240	Yellow	Round	Flat	Positive	MA	SD
SD-243	White	Round	Raised	Positive	MA	SD
SD-250	Red	Irregular	Raised	Positive	NGF	SD
SD-266	Yellow	Round	Raised	Positive	MA	SD
SD-295	Beige	Round	Raised	Positive	MA	SD
SC-2	Tan	Round	Raised	Positive	MA	SC
SC-3	White	Round	Raised	Positive	MA	SC
SC-6	White	Round	Raised	Positive	NA	SC
SC-8	White	Round	Raised	Positive	NA	SC
SC-10	White	Round	Flat	Positive	NA	SC
SC-11	Colourless	Irregular	Raised	Positive	NGF	SC
SC-13	Tan	Round	Raised	Positive	MA	SC

Isolate ID	Colony colour	Morphology	Elevation	Gram-stain	Isolation medium	Source of isolate
SC-14	Beige	Round	Flat	Positive	SWA	SC
SC-25	Grey	Oval	Raised	Positive	MA	SC
SC-26	Yellow	Oval	Raised	Positive	MA	SC
SC-43	Pink	Oval	Raised	Positive	MA	SC
SC-44	Red	Round	Raised	Positive	NGF	SC
SC-47	Colourless	Round	Raised	Positive	TSW	SC
SC-50	White	Round	Flat	Positive	MA	SC
SC-52	Red	Round	Raised	Positive	NGF	SC
SC-53	Beige	Round	Flat	Positive	MA	SC
SC-83	Red	Irregular	Raised	Positive	NGF	SC
SC-85	Beige	Round	Flat	Positive	YME	SC
SC-90	Beige	Round	Raised	Positive	MA	SC
SC-95	Beige	Oval	Raised	Positive	YPSS	SC
SC-98	White	Round	Raised	Positive	YPSS	SC
SC-100	White	Oval	Raised	Positive	MA	SC
SC-109	White	Round	Raised	Positive	MA	SC
SC-32	Beige	Round	Convex	Positive	NGF	SC
SC-33	Beige	Irregular	Raised	Positive	NGF	SC
SC-36	Brown	Round	Flat	Positive	NGF	SC
SC-42	Orange	Round	Flat	Positive	NGF	SC
DA-7	Brown	Round	Convex	Positive	NGF	DA
DA-11	Beige	Round	Raised	Positive	MA	DA
DA-19	Beige	Round	Raised	Positive	MA	DA
DA-25	Beige	Oval	Flat	Positive	SWA	DA
DA-30	White	Oval	Raised	Positive	MA	DA
DA-32	White	Oval	Flat	Positive	MA	DA
DA-37	White	Round	Raised	Positive	NA	DA
DA-38	Orange	Round	Convex	Positive	NA	DA
CR-2	Beige	Round	Raised	Positive	MA	CR
CR-5	Beige	Round	Raised	Positive	MA	CR

Isolate ID	Colony colour	Morphology	Elevation	Gram-stain	Isolation medium	Source of isolate
CR-10	Beige	Round	Raised	Positive	NGF	CR
AP-1	Beige	Round	Raised	Positive	MA	AP
AP-2	Beige	Round	Flat	Positive	MA	AP
AP-7	Beige	Round	Raised	Positive	NA	AP
AP-10	Beige	Round	Raised	Positive	NA	AP
AP-11	White	Round	Raised	Positive	NA	AP
AP-20	White	Round	Raised	Positive	NA	AP
AP-24	White	Round	Raised	Positive	MA	AP
AP-28	Yellow	Irregular	Raised	Positive	MA	AP
AP-30	White	Round	Raised	Positive	NGF	AP
AP-50	White	Round	Convex	Positive	NGF	AP
AP-57	Tan	Round	Convex	Positive	NGF	AP
AP-63	White	Round	Raised	Positive	YPSS	AP
AP-80	White	Round	Flat	Positive	YPSS	AP
AP-110	Beige	Oval	Flat	Positive	YME	AP
AP-111	Beige	Oval	Raised	Positive	YME	AP
AP-113	Beige	Oval	Raised	Positive	NGF	AP
AP-120	Beige	Round	Raised	Positive	NGF	AP
AP-139	Beige	Round	Flat	Positive	MA	AP
AP-145	Beige	Round	Raised	Positive	MA	AP
CC-9	Beige	Round	Raised	Positive	NA	CC
CC-13	Beige	Round	Raised	Positive	MA	CC
CC-14	Beige	Round	Raised	Positive	MA	CC
CC-15	White	Round	Raised	Positive	NA	CC
CC-42	White	Oval	Flat	Positive	NA	CC
CC-45	White	Oval	Flat	Positive	NGF	CC
CC-67	White	Round	Raised	Positive	NGF	CC
CC-81	Tan	Oval	Raised	Positive	NGF	CC
CC-82	White	Oval	Raised	Positive	NGF	CC
CC-83	White	Round	Convex	Positive	MA	CC

Isolate ID	Colony colour	Morphology	Elevation	Gram-stain	Isolation medium	Source of isolate
PJ-12	Orange	Round	Raised	Positive	MA	PJ
PJ-27	Tan	Round	Raised	Positive	NGF	PJ
PJ-31	White	Round	Flat	Positive	MA	PJ
PJ-38	White	Round	Raised	Positive	MA	PJ
PJ-39	White	Round	Raised	Positive	NA	PJ
PJ-40	White	Round	Raised	Positive	NA	PJ
PJ-45	Beige	Round	Raised	Positive	NA	PJ
PJ-49	Beige	Round	Raised	Positive	NA	PJ
PJ-58	White	Round	Raised	Positive	SWA	PJ
PJ-62	White	Round	Raised	Positive	NA	PJ
PJ-66	Beige	Oval	Raised	Positive	NGF	PJ
PJ-70	Beige	Oval	Raised	Positive	NGF	PJ
PJ-71	White	Round	Flat	Positive	NGF	PJ
PJ-72	White	Round	Flat	Positive	YME	PJ
PJ-73	Beige	Oval	Flat	Positive	MA	PJ
PJ-80	Beige	Oval	Flat	Positive	YME	PJ
PJ-100	Beige	Oval	Flat	Positive	YME	PJ
PJ-102	Beige	Round	Raised	Positive	YPSS	PJ
PJ-109	Beige	Round	Raised	Positive	YPSS	PJ
PJ-111	Red	Irregular	Raised	Positive	NGF	PJ
PJ-121	Pink	Round	Raised	Positive	NA	PJ
PJ-130	Beige	Round	Raised	Positive	NGF	PJ
PJ-135	Beige	Round	Raised	Positive	NGF	PJ
PJ-142	Beige	Round	Raised	Positive	NGF	PJ
PJ-149	Beige	Round	Raised	Positive	NGF	PJ